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Naive, memory and regulatory T lymphocytes populations analysis

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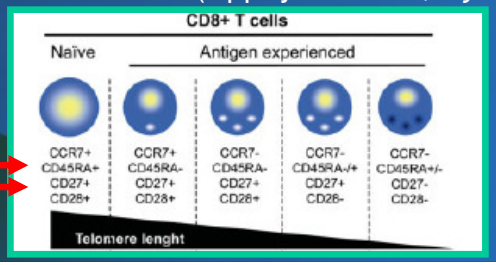
Introduction

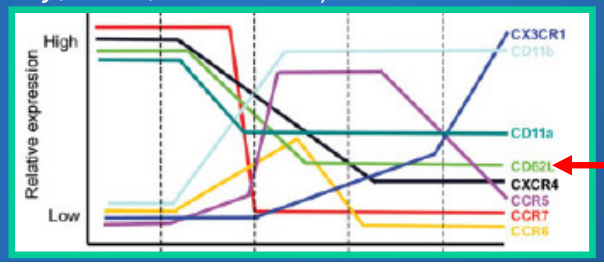
- Flow cytometric analysis of TCD4 and TCD8 lymphocytes permit identification of several sub population in HIV patients (Chattopadhyay PK and al, *Current Protocols In Immunology* 2005;12.12.1-15)
- The exploration of CD45RA, CD45R0, CD27, CD28, CD62L, CD57, CD11a, CD127, CD197 allow the identification of different maturation subsets (Chattopadhyay PK and al, *Cytometry*, 2010;77A:614-22)
- In order to identify naives and memory T cells subsets, we need to evaluate, at least, 3 markers (De Rosa SC and al, *Nat Med*, 2001;7:245-8)
- The use of multicolor flow cytometry allow us to discriminate more and more rare populations
- Rare events, which frequency is less than 1% of total events, require a viability dye such as 7-AAD or propidium iodide (Perfetto SP and al, *Nat. Rev. Immunol*, 2004;4:648-55)

- Naives T cells circulate in the lymphatic network through the HEV (High Endothelial Venuls), in which they interact with endothelials cells with their CD62L molecules (Sallusto F and al, *Nature*, 2000;401:708-12)
- T cell are activated through TCR engagment by HLA molecules, and through the link of CD28 to B7 familly molecules (CD80-86) which are expressed by Antigen Presenting Cells (Banchereau J and al, *Nature*, 1998;392:245-52)
- The activation signaling pathway is partly mediated by the CD45RA, and the CD27, and it induces proliferation of the cell, at the end (Berard M and al, *Immunology*, 2002;106:127-38)
- This activation is correlated to the acquisition of memories characters by the cell, such as the loss of CD45RA expression, in favor of the low molecular weight isoform CD45R0 (Berard M and al, *Immunology*, 2002;106:127-38)

Introduction

- The activated lymphocyte becomes effector. There are severals subtypes : Th1, Th2, Th3, iTreg, Th9, Th17, Th22, LTc. Those cells express activation molecules such as CD25, CD69, HLA-DR, CD38, some chemokines receptors (CXCR3, CCR5, CCR4, CCR3, CCR6, CCR10) and they secrete cytokines (IFN-γ, IL-4, IL-10, IL-9, TGF-β, IL-1β, IL-17, IL-22). The cytotoxic lymphocytes express and secrete perforin and granzymes molecules (Chattopadhyay PK and al, *Current Protocols In Immunology* 2005;12.12.1-15; Annunziato and al, *Arthritis Res Ther*, 2009:11:257-64)
- The use of anti-CD45R0, anti-CD45RA and anti-CD62L allow the identification of naive population which is characterized by this phenotype : $CD45R0^{-}/CD45RA^{+}/CD62L^{+}$ (De Rosa SC and al, *Nat Med,* 2003;9:112-7)
- The $CD45R0^{+}/CD45RA^{-}/CD62L^{+}$ phenotype is the classical memory profile of T cells (De Rosa SC and al, *Nat Med,* 2003;9:112-7)
- Nevertheless, the use of anti-CD27 offers the possibility to increase the identification of several subtypes, notably in the CD8 population (De Rosa SC and al, *Nat Med*, 2001;7:245-8)
- Naives T cells are $CD45RA^{+}/R0^{-}$, $CD62L^{+}$, $CD27^{+}$, central memory T cells are $CD45RA^{-}/R0^{+}$ $CD62L^{+}$ $CD27^{+}$ (Chattopadhyay PK and al, *Cytometry*, 2010;77A:614-22)
- Effector memory T cells are not clearly defined because their CD62L, CD27 expression should change with their senescence and functionnality. Some described those cells as $CCR7^{-}$, $CD28^{-}$, $CD62L^{-}$ (Chattopadhyay PK and al, *Cytometry*, 2010;77A:614-22)
- The difficulty is that for several markers, the expression changes with viral encounter for CD8 T cell (Appay V and al, *Cytometry*, 2008;73A:975-83)





The use of CD45RA/R0-CD62L-CD27 is interesting because they are early, intermediate and late antigens

Introduction

- Flow Cytometry approach permits the identification of several maturation and functionnal subsets (Annunziato and al, *Arthritis Res Ther*, 2009:11:257-64)
- Th17 population is clearly implicated in fungus and extracellular bacterial clearance (Annunziato F and al, *International Immunology*, 2008;20:1361-68)
- This population is clearly involved in several diseases such as rheumatoid arthritis, Crohn Disease, multiple sclerosis and psoriasis (Annunziato F and al, *International Immunology*, 2008;20:1361-68)
- The counter part of the Th17 cells, is a well described regulatory T cell population, called Treg
- This population is $CD3^{+}CD4^{+}CD25^{bright}CD127^{low}Foxp3^{+}$ and display suppressive capacities (For Review : *Eur J Immunol*, 2008;38:901-937)
- Those cells are involved in regulation of immune responses during infection (Belkaid Y, *Eur J Immunol*, 2008;38:918-921)
- Further more, blood Treg from rheumatoid arthritis, myasthenia gravis, psoriasis, type 1 diabetese and multiple sclerosis present default in their suppressive capacities (Costantino CM and al, *Eur J Immunol*, 2008;38:921-924)

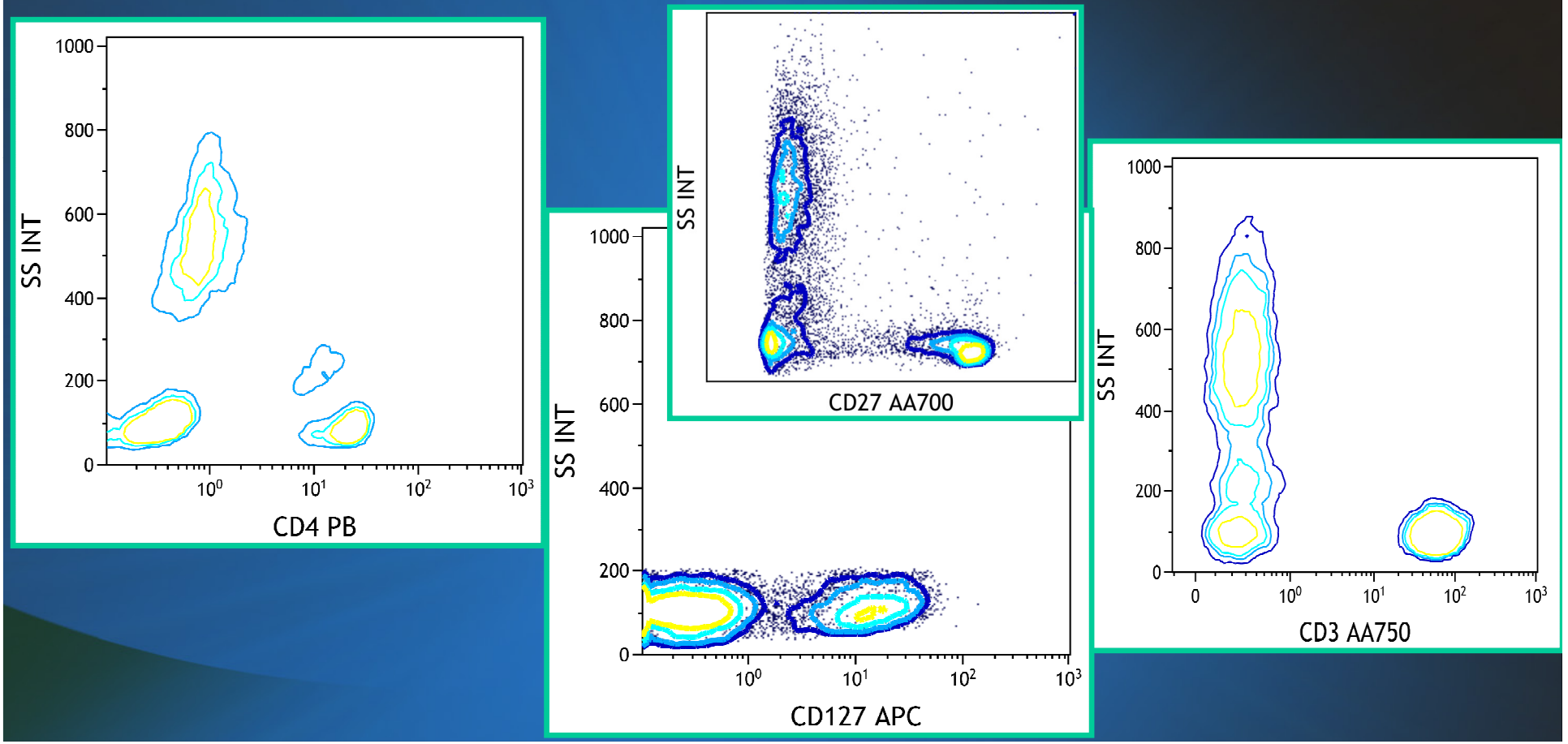
- Goal : Make a 10 colors tube to explore naive, central memory, effector memory and regulatory T cells populations

Protocol

- 100µl of peripheral blood were stained with anti CD45R0-FITC (IM1247U), anti CD45RA-PE (IM1834U), anti CD62L-ECD (IM2713U), anti CD25-PC7 (A52882), anti CD127-APC (CDS), anti CD27-AA700 (CDS), anti CD3-AA750 (A94680), anti CD4-PB (A82789) and anti CD8 KrOr (CDS)
- Dead cells were excluded with 7 AAD (A07704)
- Sample was incubated for 15 minutes, @ room temperature in the dark
- Erythrocytes were lysed by 1 ml of Versalyse (A09777), 10 minutes @ room temperature
- 2ml of PBS were added, and then tubes were centrifuged during 5 minutes, @ 20 degrees, @ 300g
- Pelets were resuspended with 500µl of PBS
- Samples were acquired on Navios 3 lasers (488nm, 638nm, 405nm)
- Data were analyzed with Kaluza Software

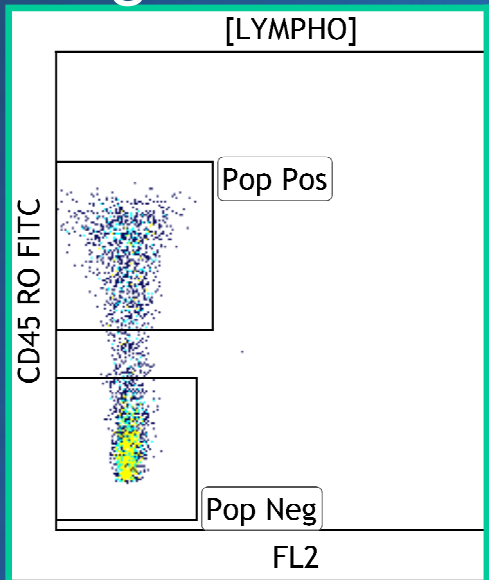
Cytometer settings

- An « All isotype tube » was done to set voltages : 100µl peripheral blood were incubated with all the isotypic controls of the staining
- In order to adjust voltages, we prepared single staining of the futur 10 colors and carefully set voltages to avoid negatives populations shift

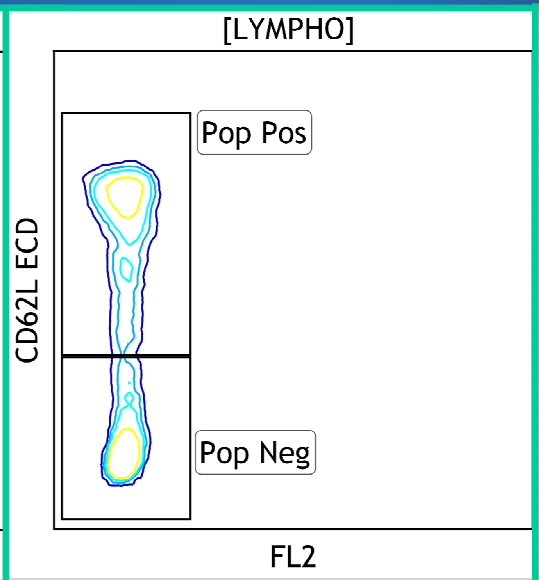


Matrix

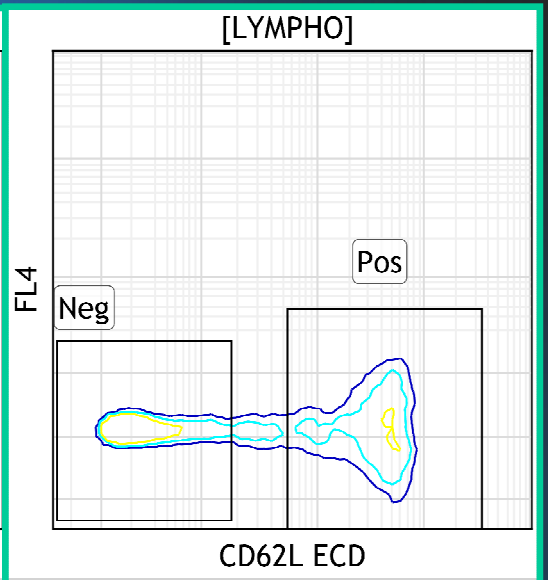
- Compensation were realized with the single color staining of the 10 colors tube, after settings voltages, in Navios Software
- Medianes Alignment were adjusted in Kaluza Logicle Mode



Gate	X-Med	X-Mean
All	0,16	0,17
Pop Neg	0,16	0,17
Pop Pos	0,16	0,16

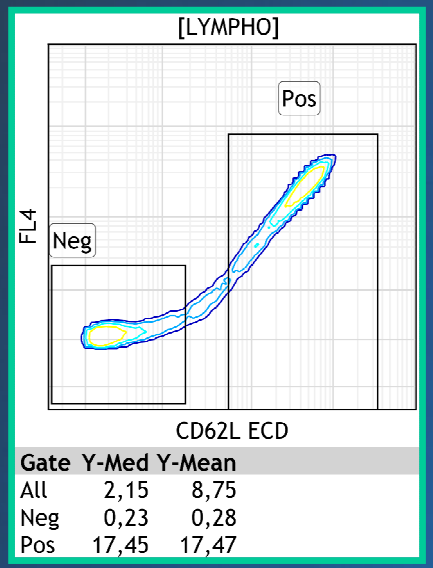


Gate	X-Med	X-Mean
All	0,15	0,16
Pop Neg	0,15	0,16
Pop Pos	0,16	0,16

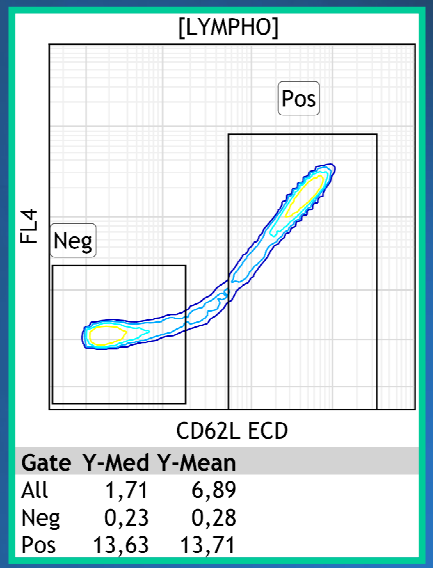


Gate	Y-Med	Y-Mean
All	0,24	0,26
Neg	0,24	0,26
Pos	0,25	0,28

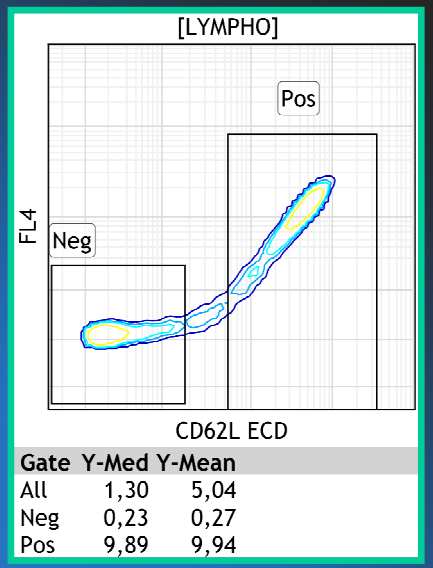
Matrix : one exemple



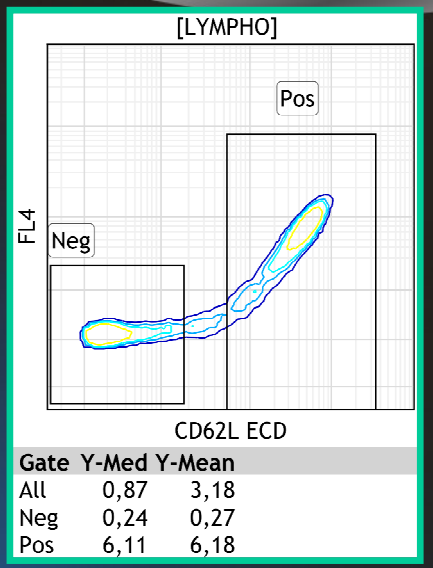
Gate	Y-Med	Y-Mean
All	2,15	8,75
Neg	0,23	0,28
Pos	17,45	17,47



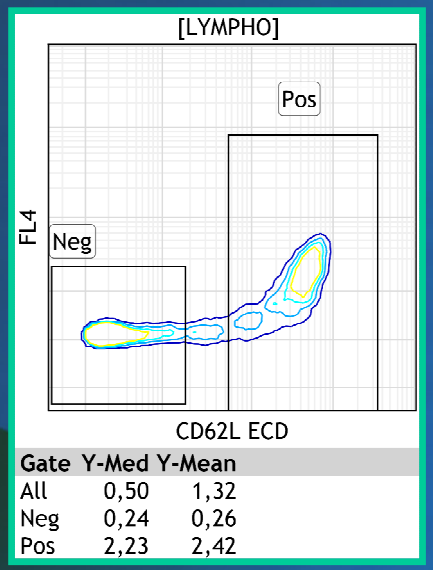
Gate	Y-Med	Y-Mean
All	1,71	6,89
Neg	0,23	0,28
Pos	13,63	13,71



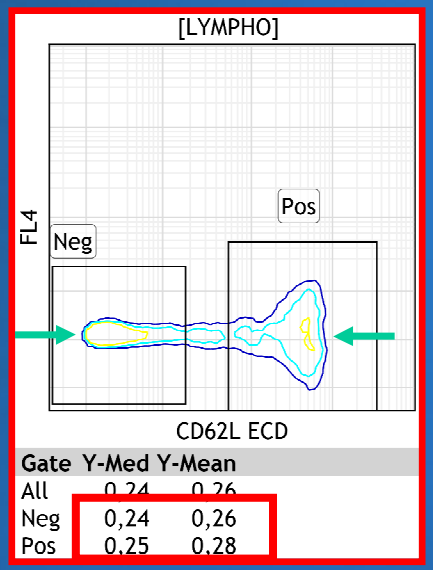
Gate	Y-Med	Y-Mean
All	1,30	5,04
Neg	0,23	0,27
Pos	9,89	9,94



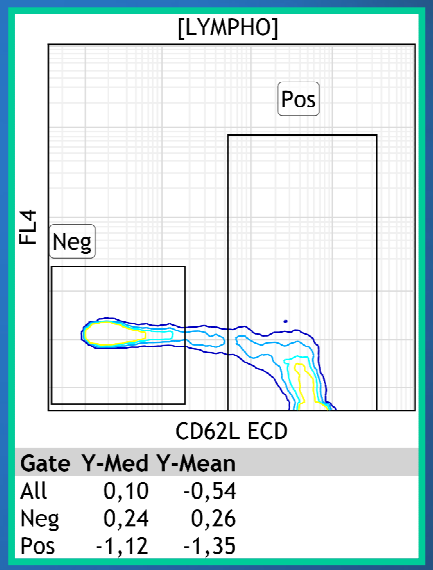
Gate	Y-Med	Y-Mean
All	0,87	3,18
Neg	0,24	0,27
Pos	6,11	6,18



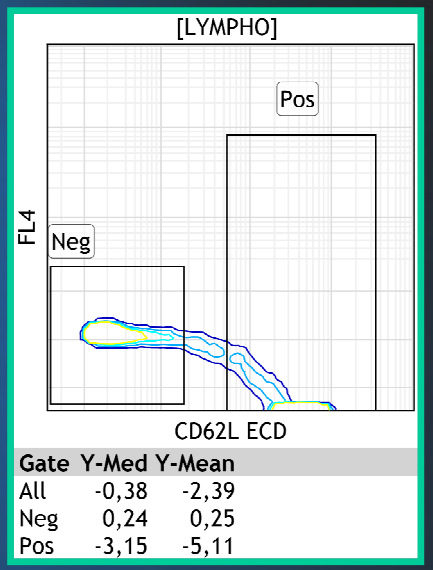
Gate	Y-Med	Y-Mean
All	0,50	1,32
Neg	0,24	0,26
Pos	2,23	2,42



Gate	Y-Med	Y-Mean
All	0,24	0,26
Neg	0,24	0,26
Pos	0,25	0,28

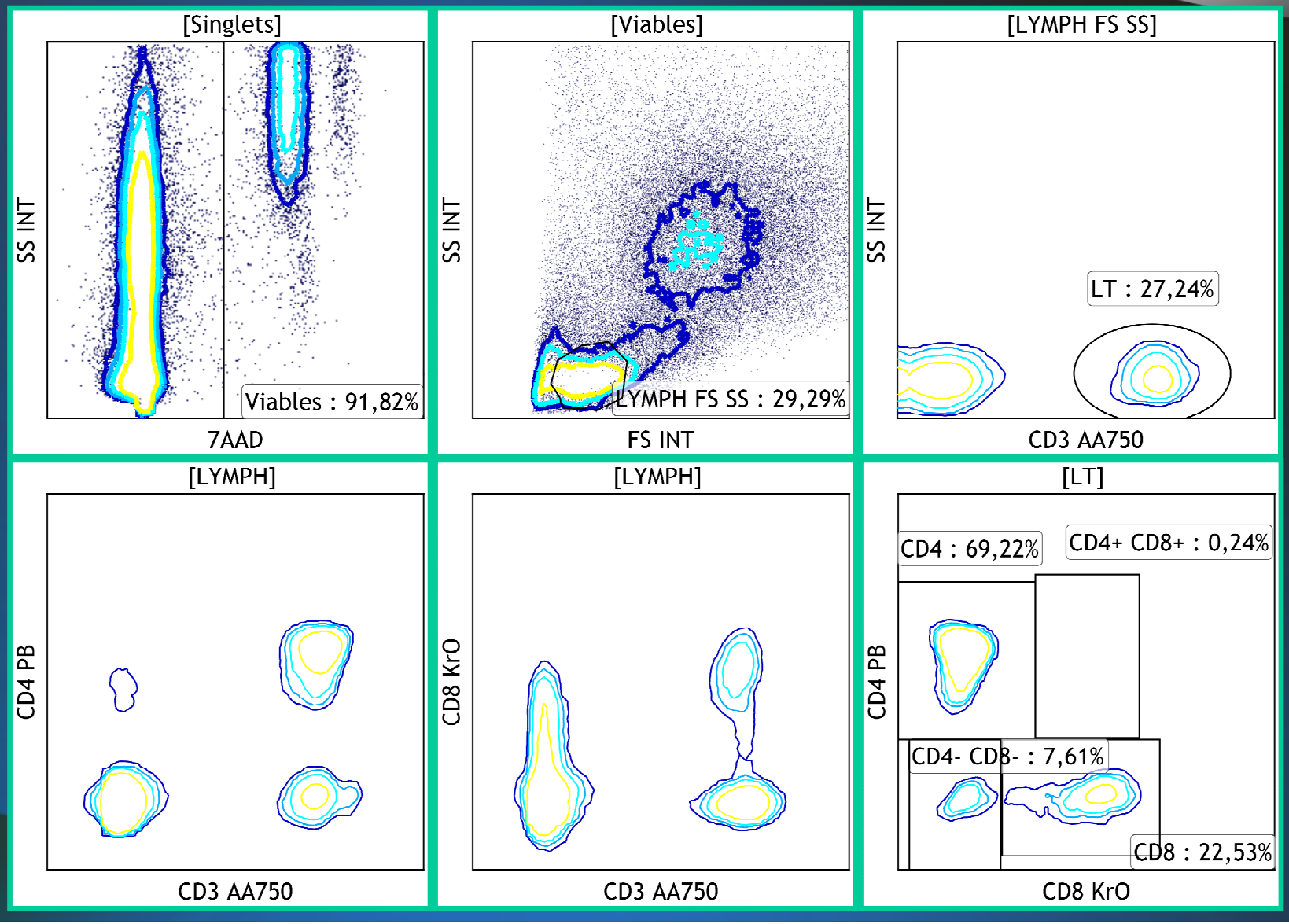


Gate	Y-Med	Y-Mean
All	0,10	-0,54
Neg	0,24	0,26
Pos	-1,12	-1,35

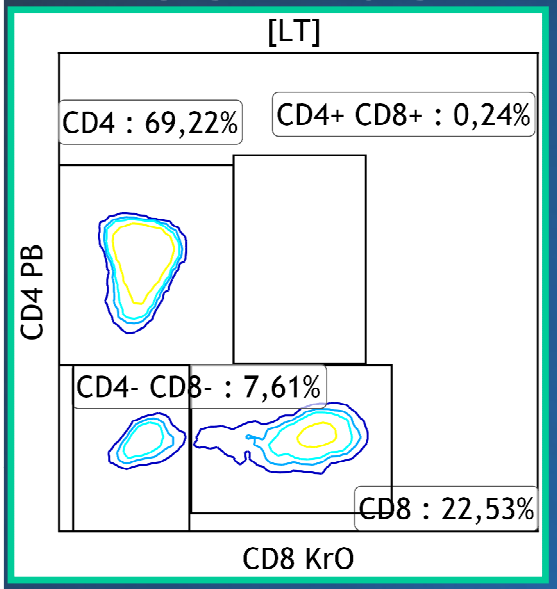


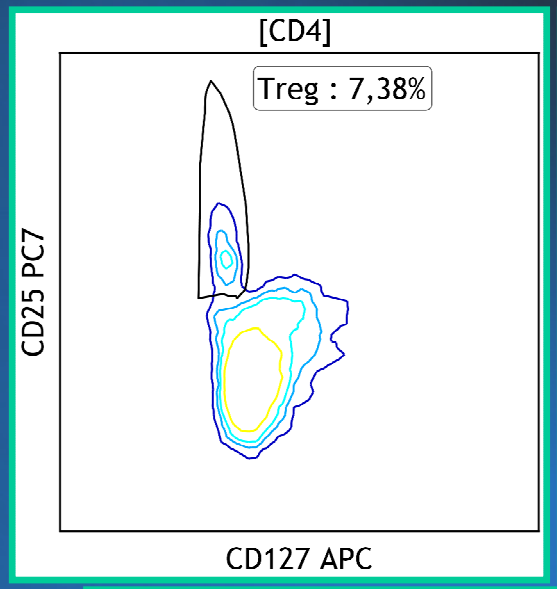
Gate	Y-Med	Y-Mean
All	-0,38	-2,39
Neg	0,24	0,25
Pos	-3,15	-5,11

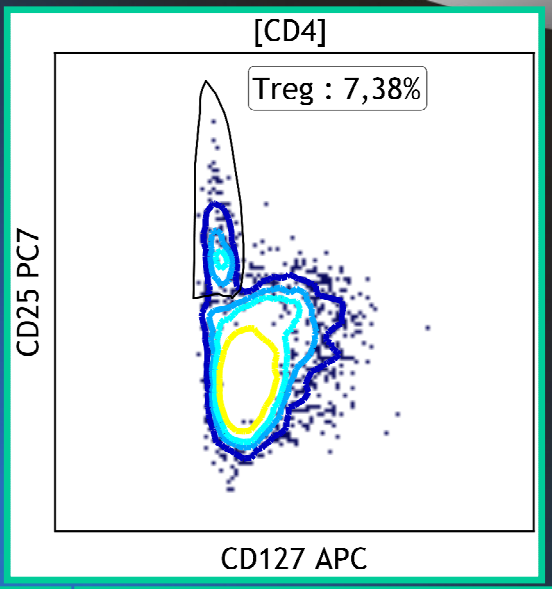
Results


[Singlets]
SS INT
7AAD
Viables : 91,82%
[Viables]
SS INT
FS INT
LYMPH FS SS : 29,29%
[LYMPH FS SS]
SS INT
CD3 AA750
LT : 27,24%
[LYMPH]
CD4 PB
CD3 AA750
[LYMPH]
CD8 KrO
CD3 AA750
[LT]
CD4 PB
CD8 KrO
CD4 : 69,22%
CD4+ CD8+ : 0,24%
CD4- CD8- : 7,61%
CD8 : 22,53%

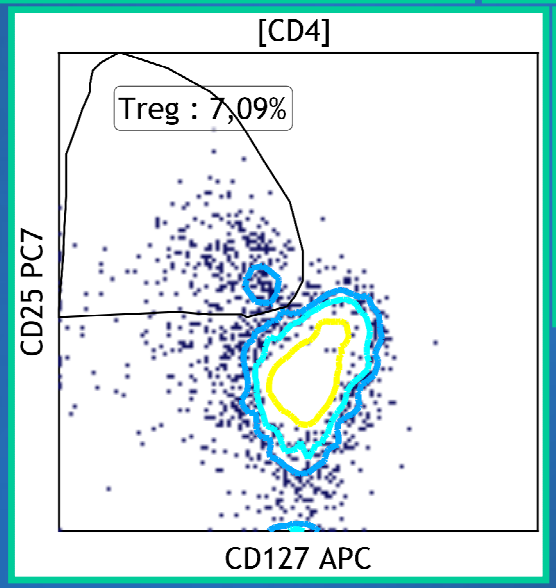
Results







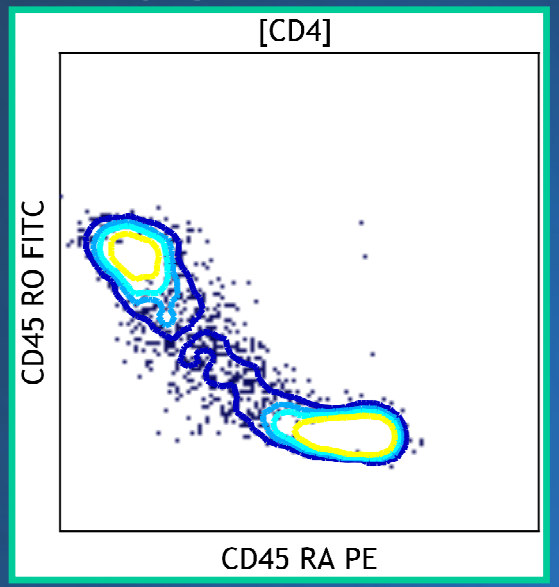
« Logicle » representation of data facilitates grouping of population

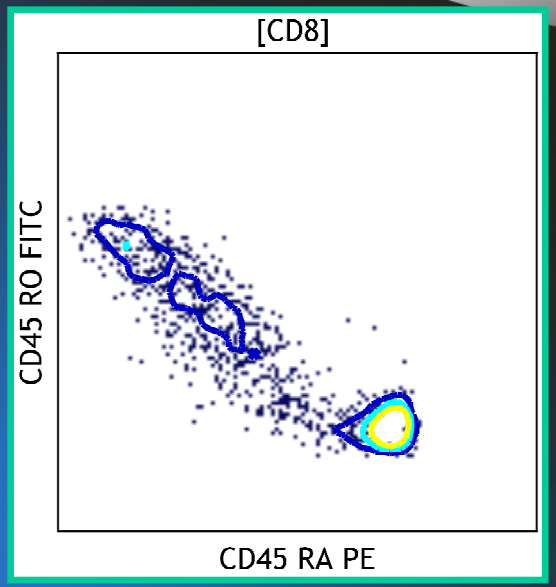


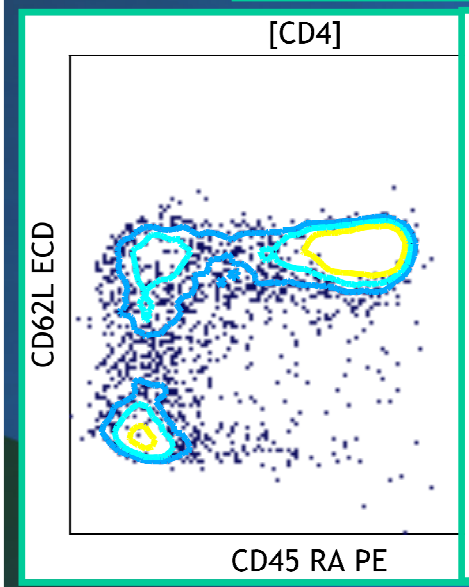
Subpopulations in T lymphocytes CD3+

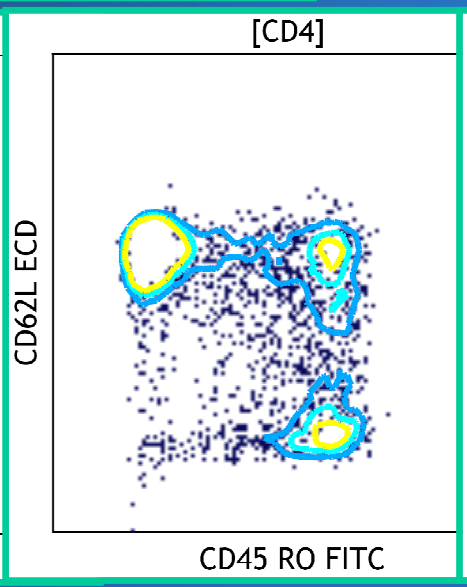
Gate	Number	%Gated
Treg	415	7,09
CD8	1 884	22,28
CD4	5 855	69,23
CD4- CD8-	666	7,88
CD4+ CD8+	19	0,22

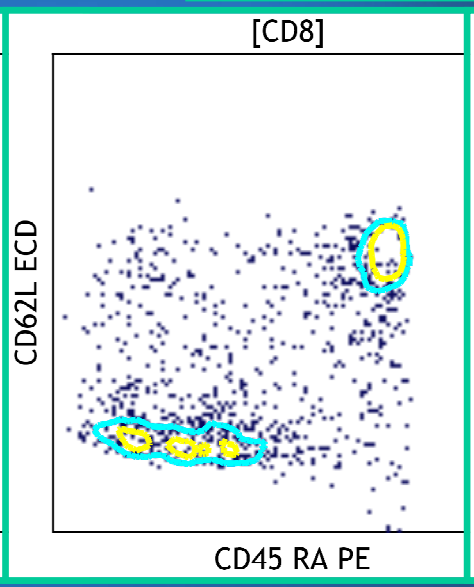
Results

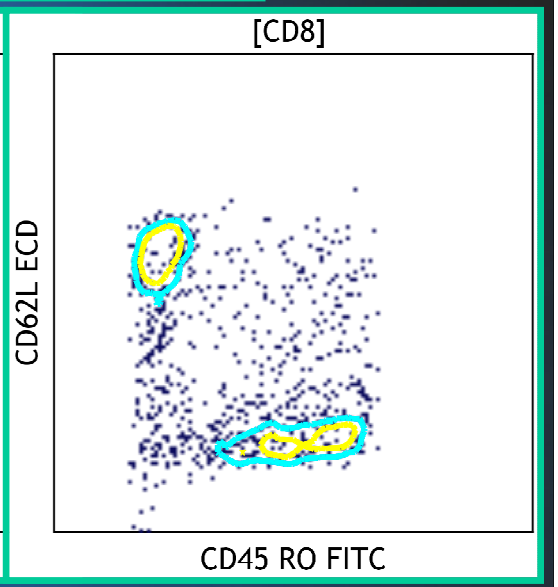

[CD4]
CD45 RO FITC
CD45 RA PE


[CD8]
CD45 RO FITC
CD45 RA PE

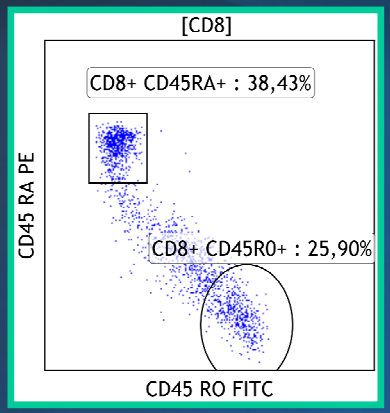

[CD4]
CD62L ECD
CD45 RA PE

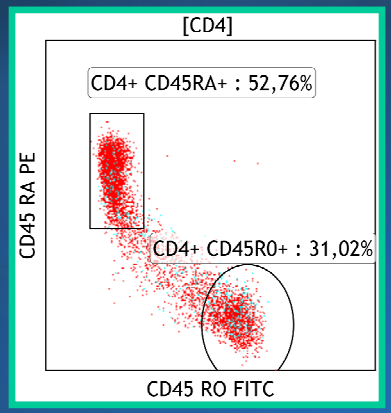

[CD4]
CD62L ECD
CD45 RO FITC

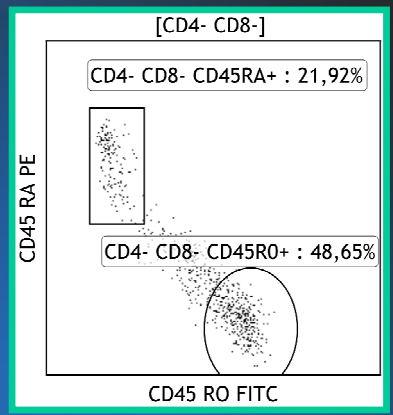

[CD8]
CD62L ECD
CD45 RA PE

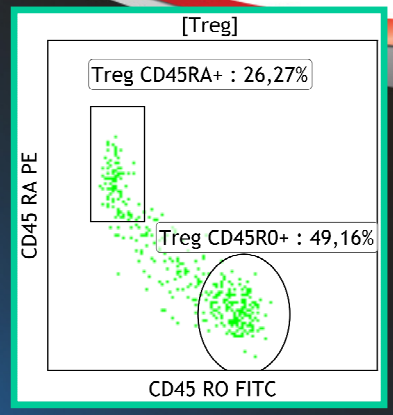

[CD8]
CD62L ECD
CD45 RO FITC

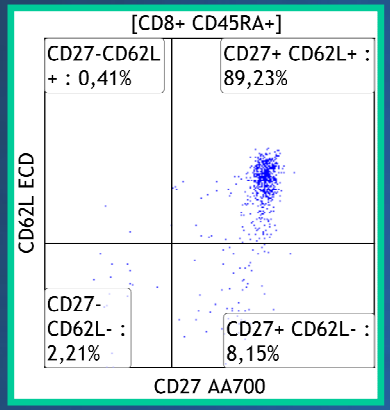
Results

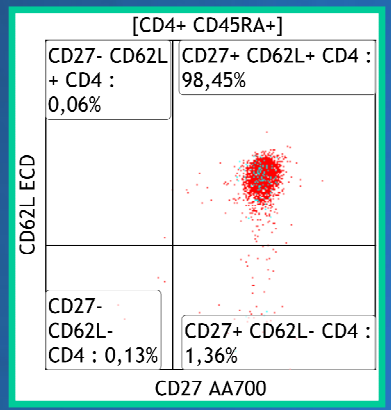


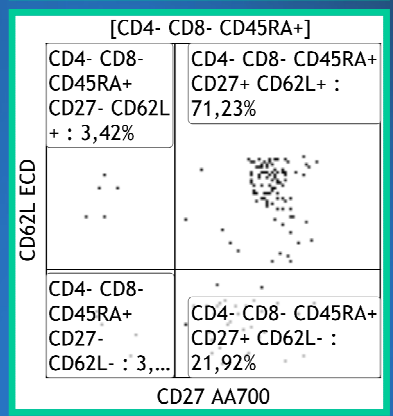


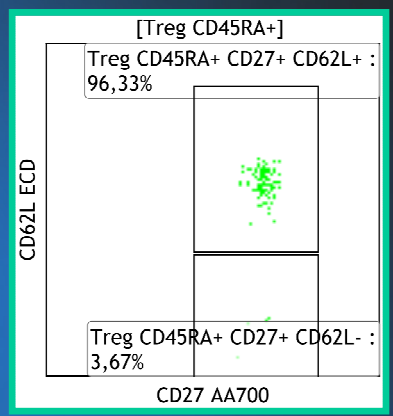




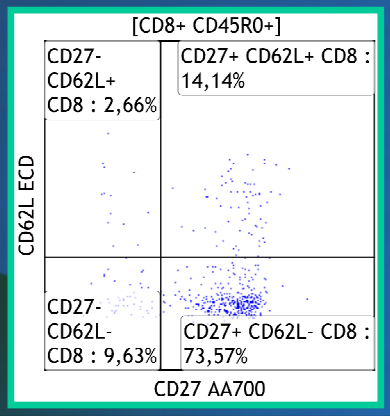


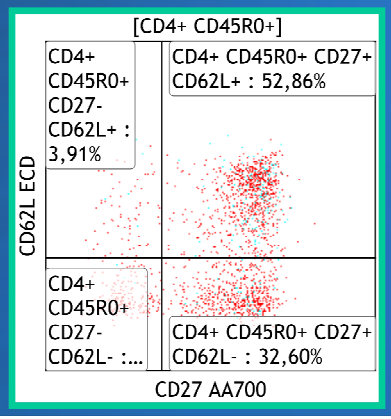


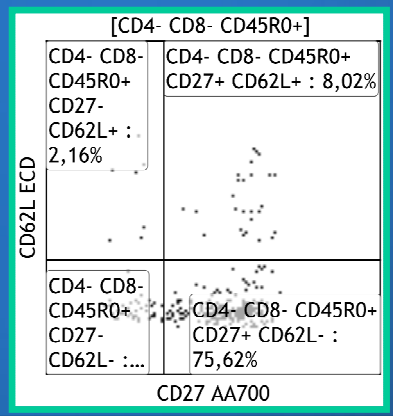


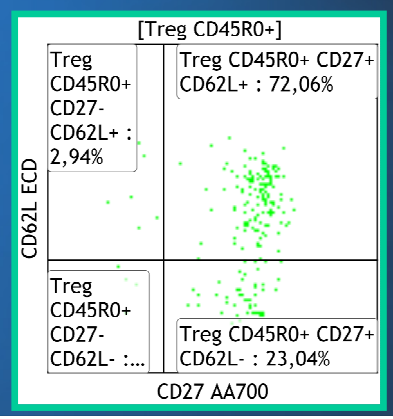


Naives cells : 45RA^{+} 62L^{+} 27^{+}





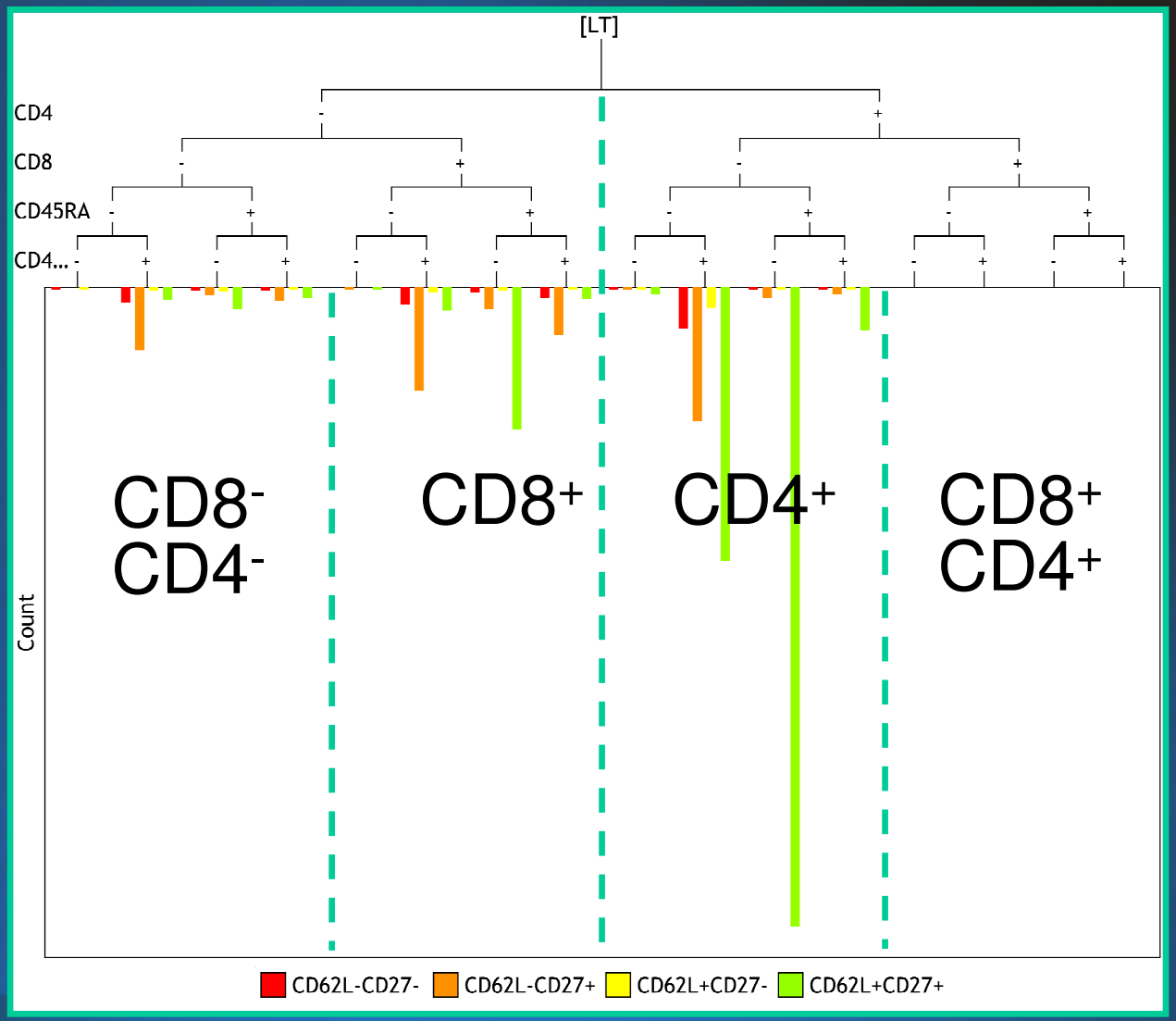




Central memory cells : 45R0^{+} 62L^{+} 27^{+}

Effector memory cells : 45R0^{+} 62L^{-} 27^{+}

Results


[LT]
CD4
CD8
CD45RA
CD4...
CD8- CD4-
CD8+
CD4+
CD8+ CD4+
Count
CD62L-CD27-
CD62L-CD27+
CD62L+CD27-
CD62L+CD27+

Conclusion

- Results can be discussed, because of lack of consensus on markers that are crucial to track naive, memory, effector and regulatory T cells
- Nevertheless, results are in concordance with some published results (Chattopadhyay PK and Roederer M, *Cytometry*, 2010;77A:614-622)
- So, for maturation study, the use of the 4 markers CD45RA/R0/CD62L/CD27 gives convincing and powerful results
- Addition of the CD57, or another, could provide a powerful understanding of the T immune system for vaccine trial (Petrovas C and al, *J Immunol*, 2009;183:1120-32; Chattopadhyay PK and Roederer M, *Cytometry*, 2010;77A:614-622)
- In order to increase the number of markers, it could be usefull to make a DUMP Channel, in order to include 7AAD, CD14 PC5.5, CD19PC5.5, CD56 PC5.5 to eliminate population that don't need to be analyzed (Perfetto SP et col, *Nat Rev Immunol*, 2004;4:648-55)

So We can Provide 1 or x (variation on FL2) tubes 10 colors for T cells exploration :

CD57PB/CD8KO/CD45R0FITC/CD45RA or CD28 or CD11a or CD25 or CCR7PE /CD62LECD/7AAD+CD19PC5.5+CD56PC5.5+CD14PC5.5/CD27PC7/CD4APC /CD127AA700/CD3AA750