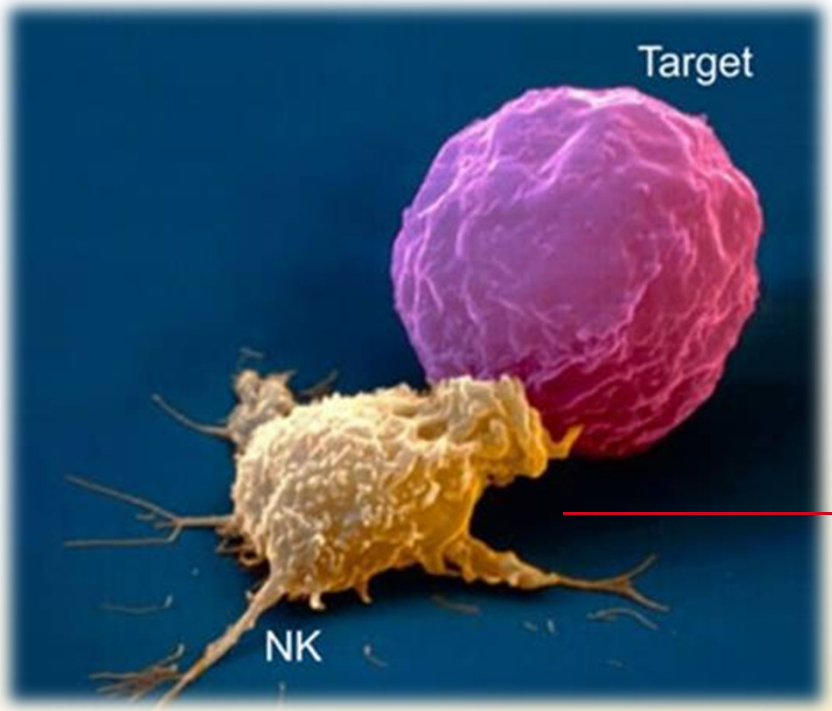

Phenotyping NK cells and NKT cells populations by Flow Cytometry

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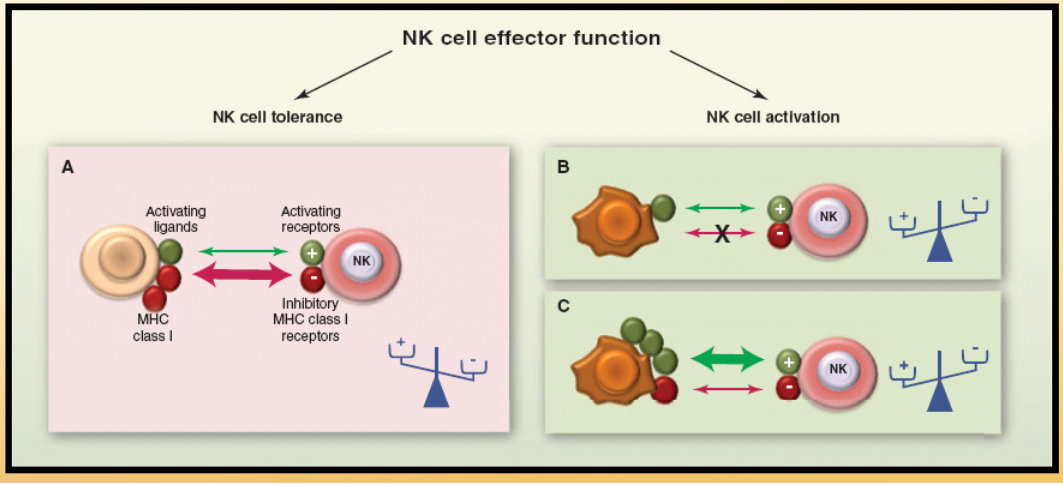
1.Introduction : NK cells

- Natural killer cells were discovered in 1975 (1)
- NK express the NCAM-1 molecule, which clusterises as CD56 (2)
- In blood, we identify at least two mains populations of NK cells, the $CD56^{dim}$ and the $CD56^{bright}$ (2)
- Another classical marker of NK cells, is the FcγRIII also called CD16 (3)
- CD16 is an activating receptor, which could generate the ADCC (Antibody Dependant Cell Cytoxicity) (4)
- CD16 expression confers to NK, the capacity to recognize opsonized cell by antibodies, and then direct killing of cells (4,5)
- Although NK cells are armed for killing, they are not dangerous in steady state conditions
- In fact, they express activating and inhibitory receptors (6)
- The resulting effect depends of the balance of engaged activating and inhibitory receptors (7)

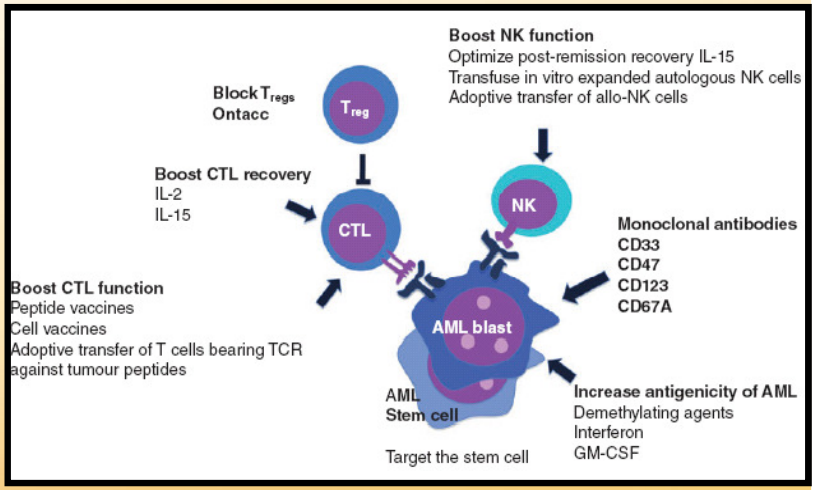


1.Introduction : NK cells

- Inhibitory receptors prevent host cells killing, some are called KIRs (Killing Inhibitory Receptors or CD158…) [8]
- KIRs recognize HLA class I molecules that prevent killing of normal cells, by an ITIM transduction dependent pathway [8,9]
- KIRs are not the only inhibitory receptors, NKG2A is an important inhibitory receptor that heterodimerizes with CD94 [10]
- Those molecules recognizes HLA-E molecule, unlike the KIRs which classically recognize HLA-A, B and C molecules [8,10,11]
- CD94 also heterodimerizes with NKG2D, which is an activating receptor when linking to MIC-A, MIC-B or ULBP [12]
- The last are cell stress induced molecules [12]
- In some cases, upregulation of NKG2D ligands is sufficient to induce NK cell mediated lysis of tumor cells [13]
- Expression evaluation of the principal KIR, NKG2A and NKG2D would be important to predict the potential effect of NK cell infusion as a way of cancer treatment [13,14]

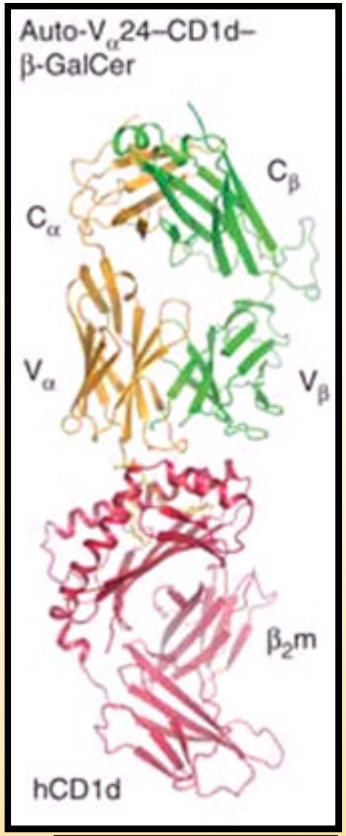
1.Introduction : NK cells

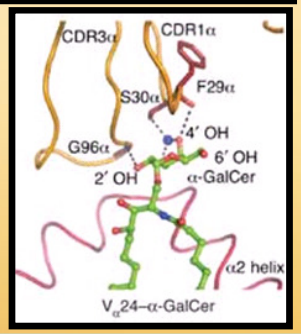
- Natural Cytotoxicity Receptors (NCRs) are very important in NK cell activation [15]
- The well known NCRs are NKp30 (CD337), NKp44 (CD336) and NKp46 (CD335) [15]
- Even if NKp ligands are not fully known, they associate with different activating co-receptor and transduce signaling that promote NK cell activation and lysis of cancer cells [13,16,17]
- Evaluation of their expression should be of interest in NK cell based or related therapy [13,14]
- NK cell related therapy have been tried in several pathologies [13] , such as acute myeloid leukemia [18,19,21]
- Several NK cell based therapy were successfull in AML [19,21]
- This success could be, in part, explained by non KIRs engagement by HLA molecules (KIR HLA mismatch), and activating receptors triggering by cancer cells ligands [14,18,19,20,22]
- This provides a positive balance that generates NK activation and killing of tumor cells called Graft Versus Leukemia [7,20,22]



1.Introduction : CD3+ CD56+ cells

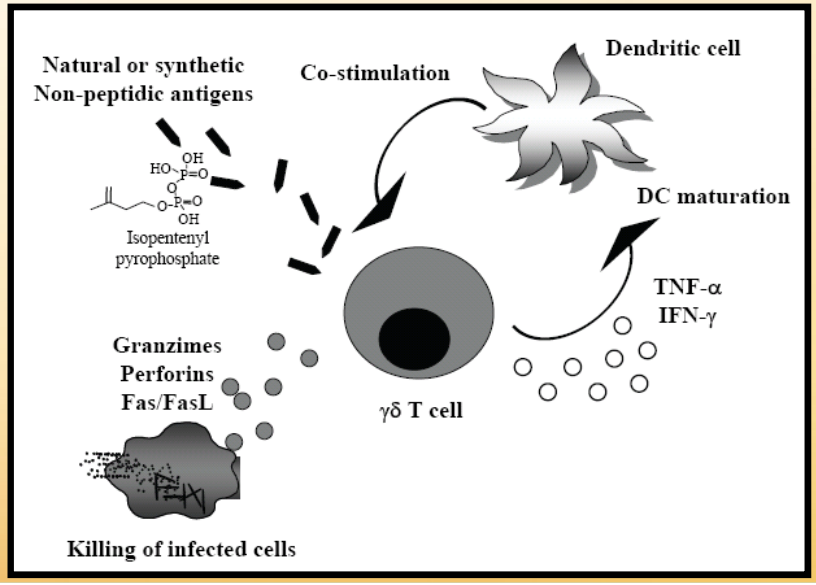
- In blood, there is at least two lymphocytes populations that express CD56 as NK cells
- Those cells are $CD3^+$ also, so this is a kind of cells that express NK marker and T cells marker, that's why some call them NKT cells
- NKT cells are a heterogenous population (23)
- Some describe two mains populations : type I and type II NKT cells (23,24)
- The first are the iNKT cells, that express the invariant TCR Vα24-Jα18 which recognizes the glycolipid αGalCer (alpha Galactosyl Ceramide) (23,24)
- The second are also CD1d restricted, but they express a range of TCRs
- They could recognize glycolipid also (25)
- Type I and Type II NKT cells are in charge of recognizing microbial lipid presented by the CD1d molecules (25,26)
- It was described that iNKT could prevent NOD mice diabetes development (27)
- Even there is some αGalCer injection assays (28,29), injection of iNKT is poorly done in human, nevertheless it seems to have anti-tumor effect (30)
- The definition of iNKT cells, is for a lot of works only the reactivity toward CD1d-αGalCer tetramer (one exemple in human : ref 31)





1.Introduction : CD3+ CD56+ cells

- NKT cells are CD3+ CD56+, but they are not the only populations that co-express these two molecules in blood
- Yδ T cells also share this co-expression
- This population is a heterogenous population that is implicated in innate immune response to tumors and pathogens (32,33)
- There is two mains populations of Vδ1 and Vδ2, in blood (32,33)
- The majority of Yδ T cells seems to be Vγ9Vδ2 (80-90% of Yδ T cells in peripheral blood)
- Yδ T cells share innate immune receptors as well as adaptive immune receptors
- They express NKG2D, NKG2A, CD94, as NK Cells (32-34)
- In contact of tumor cells or pathogens infected cells, Yδ T cells secrete pro-inflammatory cytokines, and could kill the transform target (32,33,36)
- With their Yδ TCRs, they recognize non peptidic phophorylated antigens or lipids (35-36)
- The most described Yδ T cells population is the Vγ9Vδ2, it recognizes some pyrophosphate derived products, that are essential for cell growth, membrane integrity of bacteria and mammalians cells (36-38)



1.Introduction : CD3+ CD56+ cells

- Even if immunological memory is a key mark of adaptative immunity, Yδ T cells could be differentiated into naive, central memory, effector memory and $CD45RA^+$ effector memory cells [39]
- This differentiation could be done with CD45RA, CD45R0, CD62L, CD27 or CD28 [39]
- Vγ9Vδ2 T cells are strongly activated by phospho-antigens from mycobacterium tuberculosis, whereas Vδ1 emerged signicantly in HIV and CMV infection [40]
- It seems that Yδ T cells could secrete IL17, this last has a critical role in tumors rejection and pathogens rejection [41]

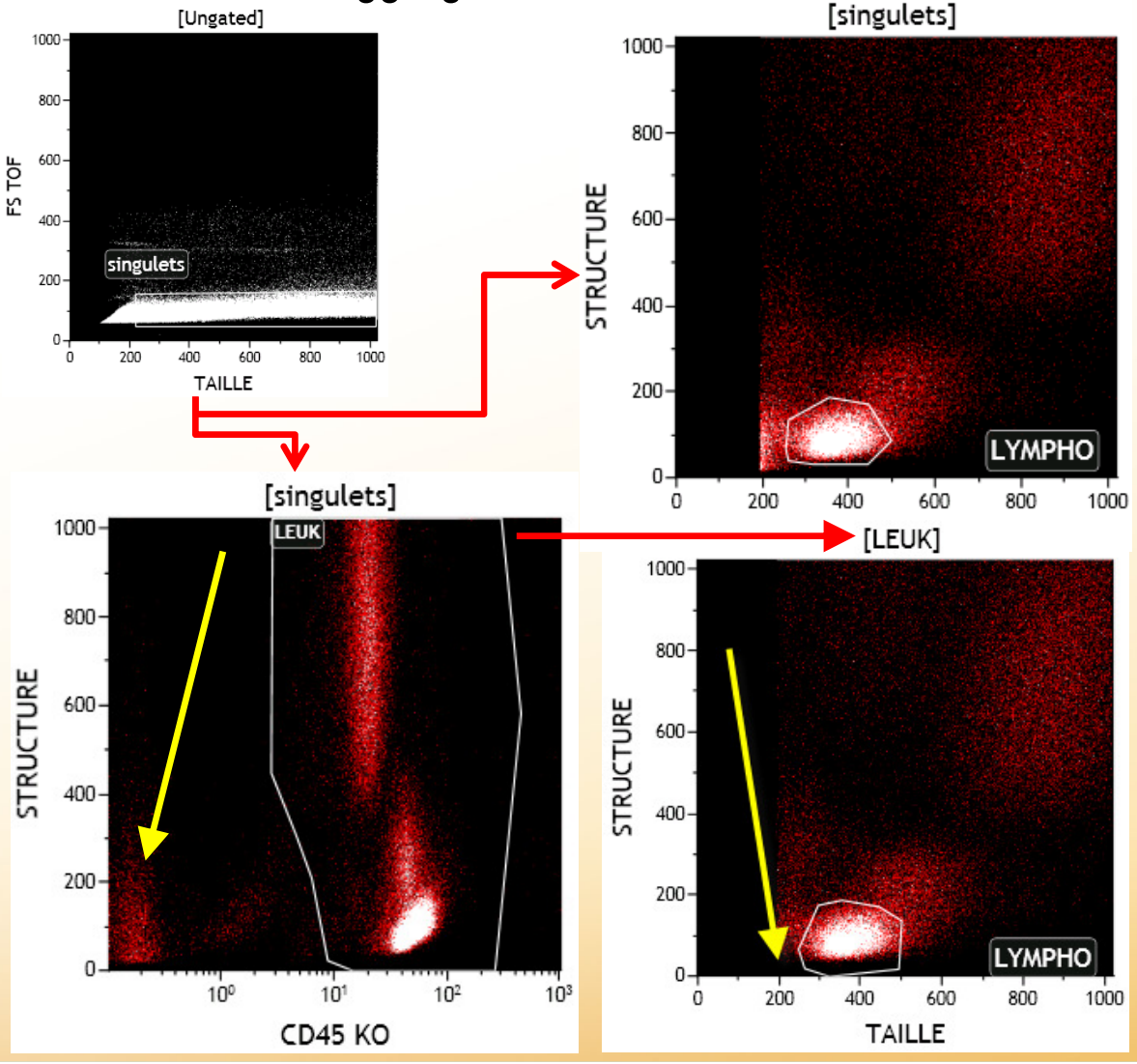
- <u>Goal</u> : the aim was to make a 10 colors tubes panel, that permit phenotyping of NK cells, NKT cells and T cells, in order to access to inhibitory and activating molecules, as well as naive and memory molecules on each cells type

2.Material and method

- Healthy donor fresh blood sample was directly stained with those antibodies
  - Pacific Blue : Anti CD57 or anti CD45RA
  - Krome Orange : anti CD45 or CD4
  - FITC : anti TCR Vα24 or anti CD45R0 or anti CD95 or anti TCR Yδ
  - PE : anti CD11b or anti Vα24 or anti NKp46 or anti CD28 or anti CD94 or anti TCR Yδ or anti NKG2D or anti NKG2A or anti CD158a,h or anti CD158b or anti CD158e1,e2 or anti CD158i or anti NKp30
  - ECD : anti CD62L or anti CD45R0 or anti NKp46
  - PC5.5 : CD56 or PC5 : anti NKp44
  - PC7 : anti CD3 or anti CD56
  - APC : anti CD38 or anti CD69 or anti CD158b1,b2,j or anti CD158 a,h or anti CD158a
  - APC-AlexaFluor 700 : anti CD16
  - APC-AlexaFluor 750 : anti CD8 or anti CD64
- Incubation was performed at room temperature during 15 minutes
- Red Blood lysis was realized with 1 ml of Versalyse per 100µl of blood
- Samples were then washed once, 1 ml PBS was added and centrifuged @ 300g during 5 min
- Pelets were resuspended with 300µl of PBS
- Acquired on Gallios 3 lasers (Blue, Red, Violet)
- Data were analyzed with Kaluza Software V1.2

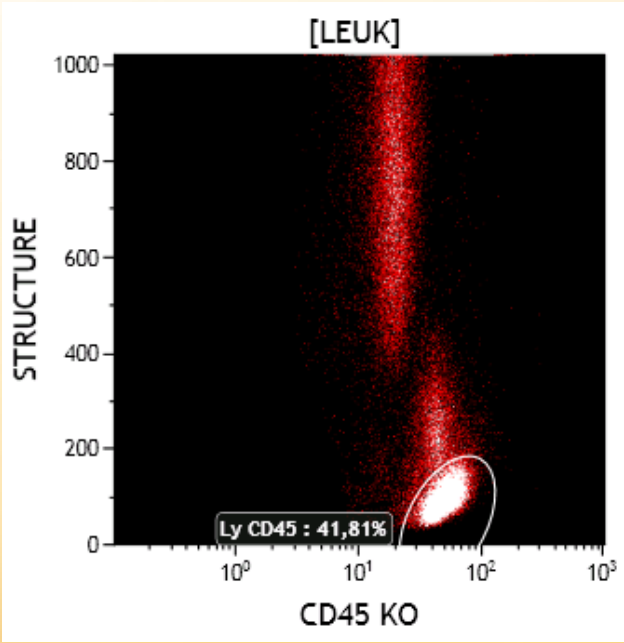
3.Results : Gating strategy to identify NK/NKT

Doublets and aggregats of cells were eliminated with a FSC Area vs FSC TOF signal

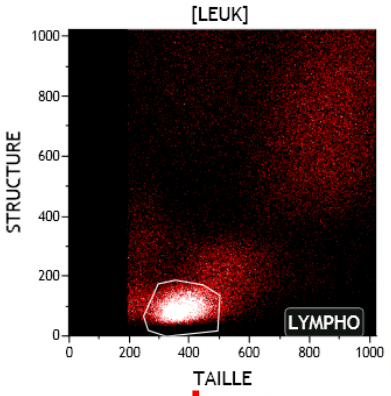


FSC vs SSC Dot plot was created to take lymphocytes

In some tubes, CD45 permits to eliminate debris, and to take all the leukocytes



3.Results : Gating strategy to identify NK/NKT



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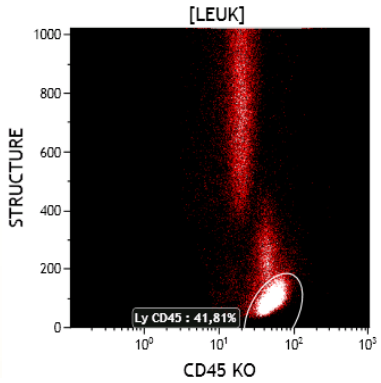
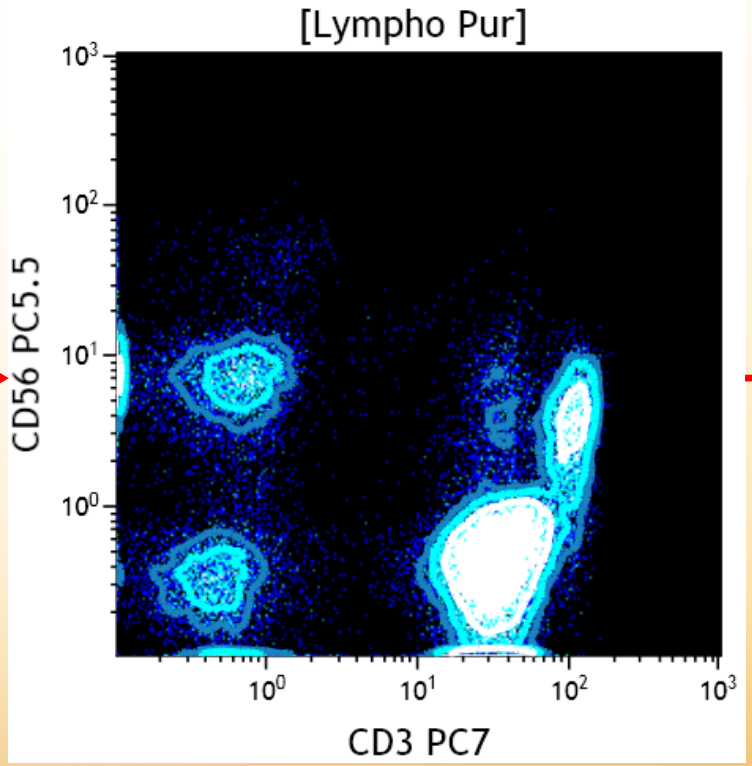
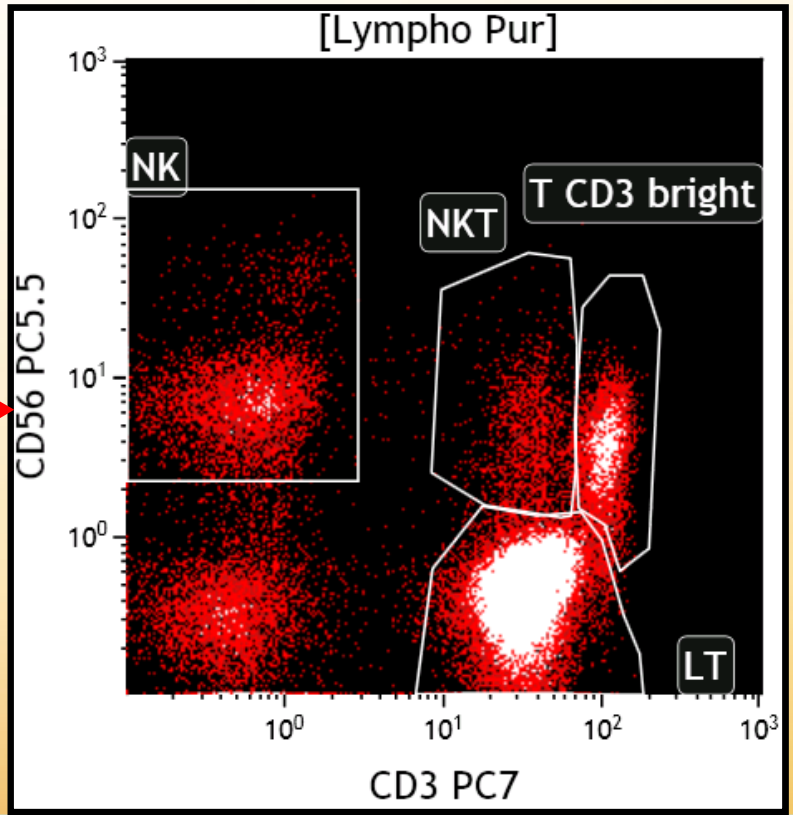


Lympho Pur gate was created as lympho and NOT Mono

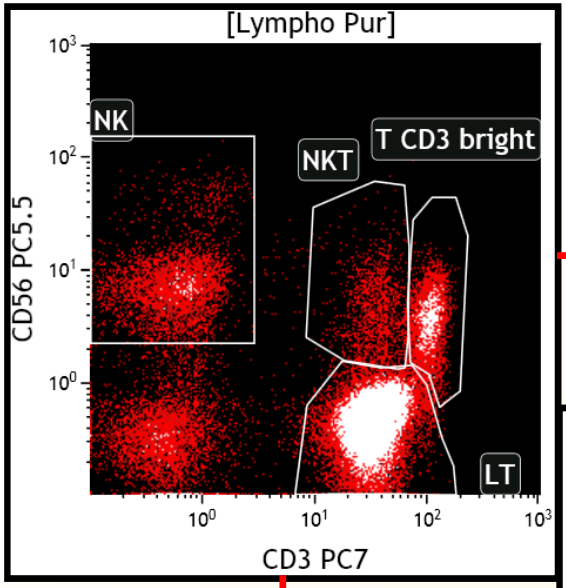
Monocytes were gated based on CD45 or CD4 Krome Orange or CD64 APC AlexaFluor750

CD3 vs CD56 Dot Plot allows the identification of $CD56^+$ $CD3^-$ NK cells, $CD56^-$ $CD3^+$ T lymphocytes, $CD3^+$ $CD56^+$ NKT and $CD3^{bright}$ $CD56^+$ probably γδ T cells





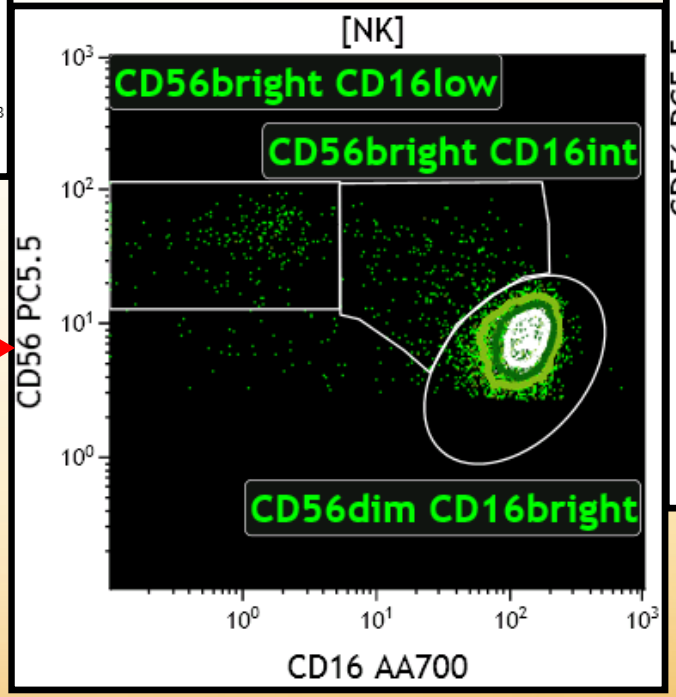
3.Results : NK populations

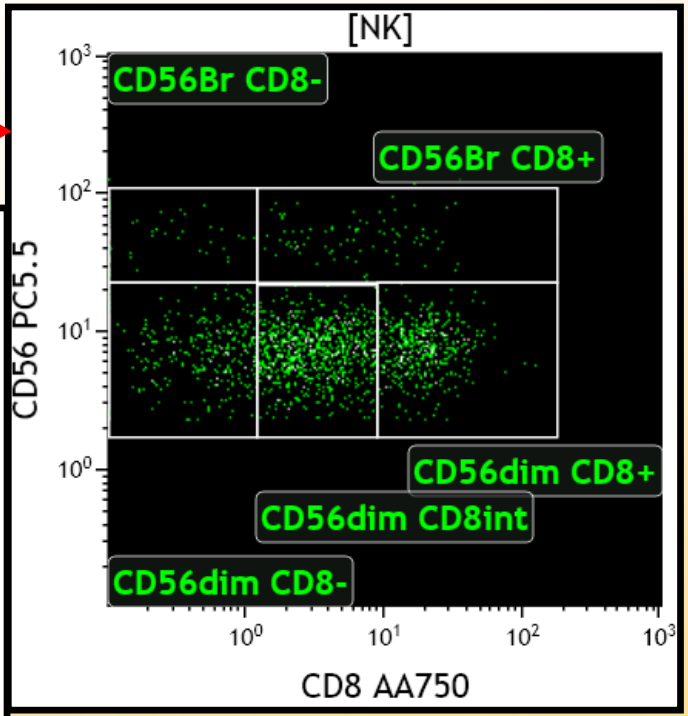


CD16 and CD8 expression on NK cells permit the identification of differents populations :

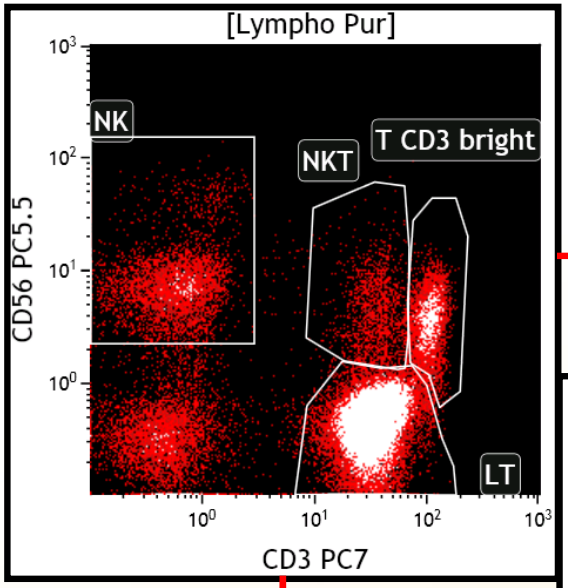
$CD56^{bright}$ $CD16^{Low}$, $CD56^{bright}$ $CD16^{Int}$,$CD56^{dim}$ $CD16^{bright}$

$CD56^{bright}$ $CD8^{-}$,$CD56^{bright}$ $CD8^{+}$,$CD56^{dim}$ $CD8^{-}$,$CD56^{bright}$ $CD8^{int}$,$CD56^{dim}$ $CD8^{+}$





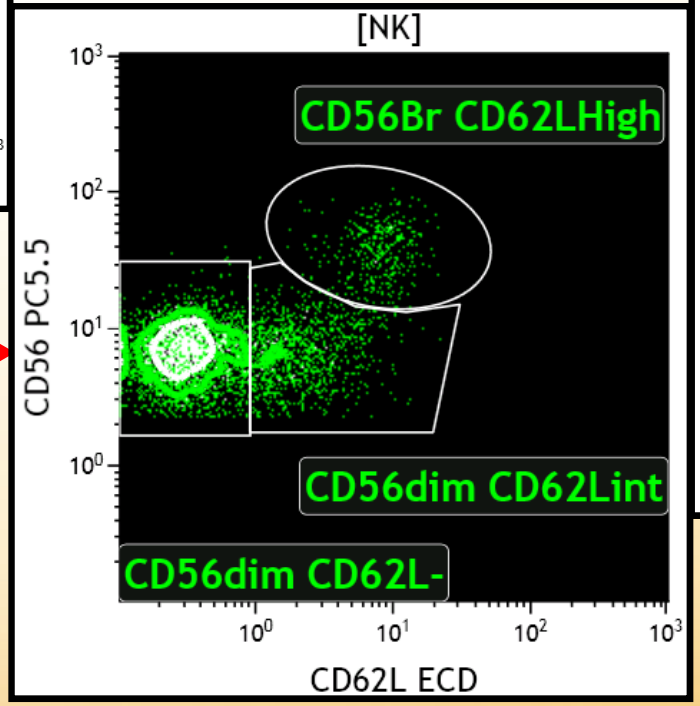
3.Results : NK Populations

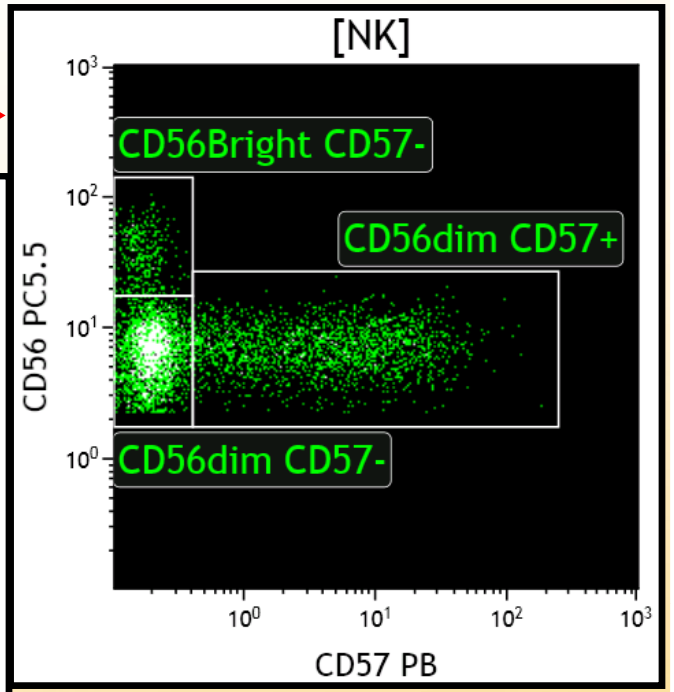


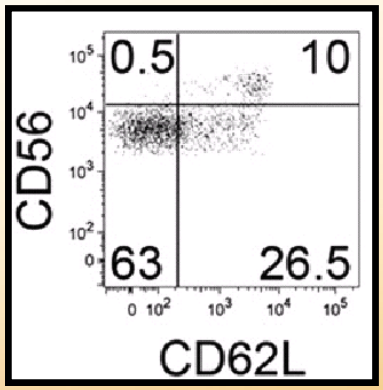
As described [42], CD62L expression on NK cells permits to identify three mains populations :

$CD56^{bright}$ $CD62L^{High}$, $CD56^{dim}$ $CD62L^{Int}$,$CD56^{dim}$ $CD62L^{-}$

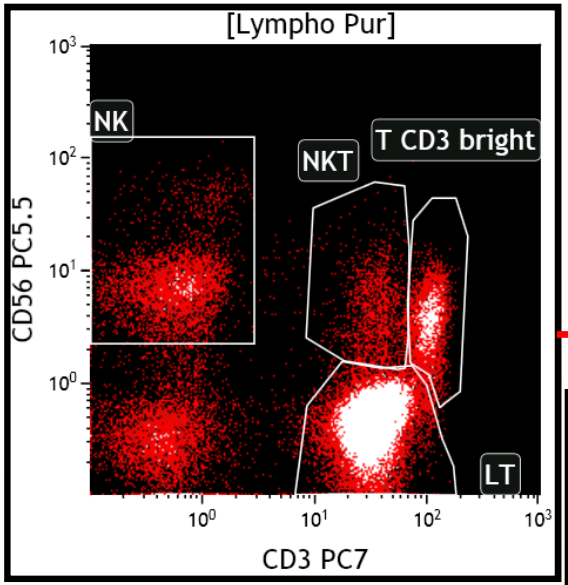
With CD57, there is also 3 mains populations $CD56^{bright}$ $CD57^{-}$,$CD56^{dim}$ $CD57^{-}$, $CD56^{dim}$ $CD57^{+}$



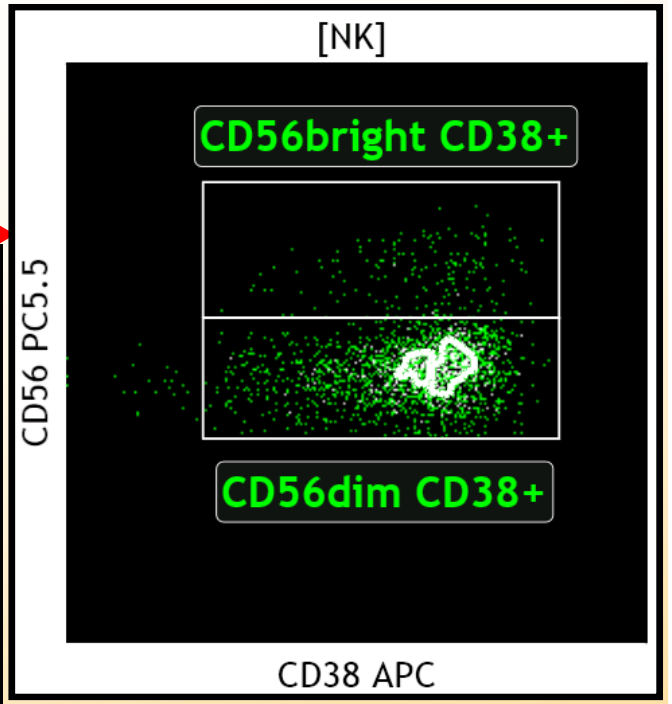




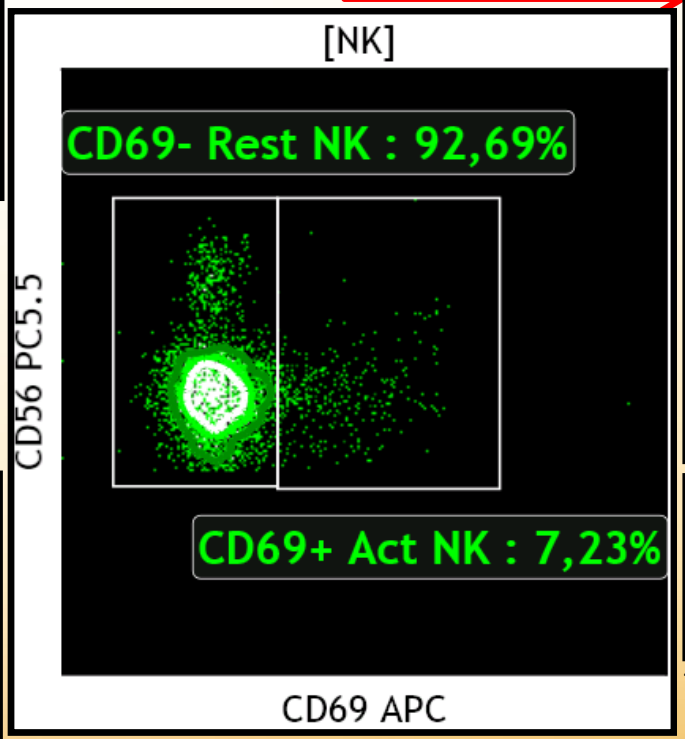
3.Results : NK Populations

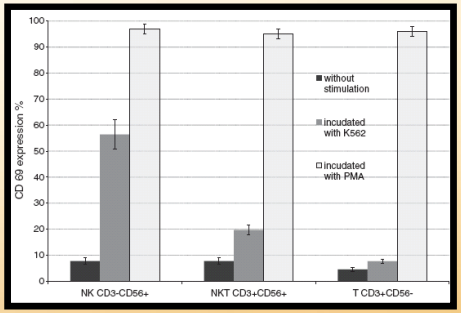


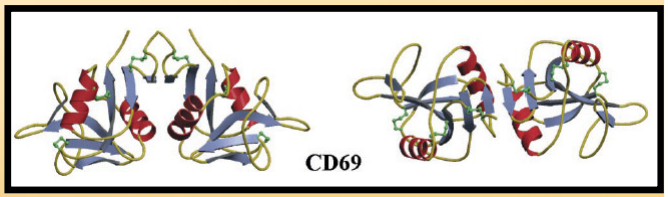
CD38 is an important signaling molecule for NK cell. Its engagement by anti CD38 conducts to increase of NK cell cytotoxicity [43,44]
Here, we identified that nearly all NK express CD38



CD69 is one of the activations markers expressed by NK cells [45]
Here, we identified very few Activated NK cell, the majority of NK cells were CD69-, so those NK cells are Resting NK cells

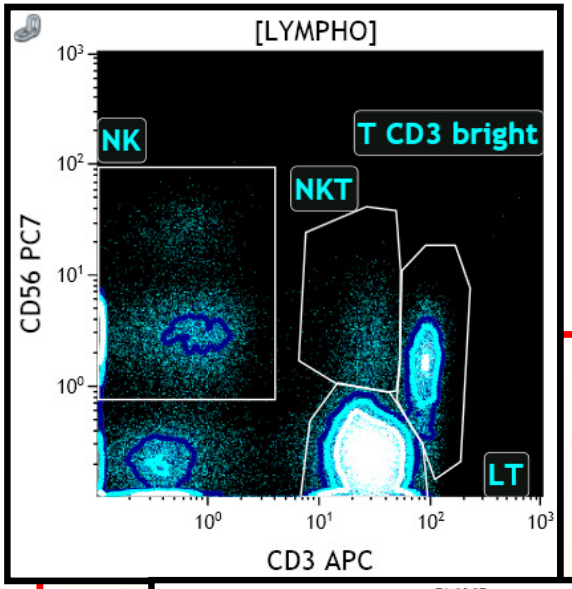




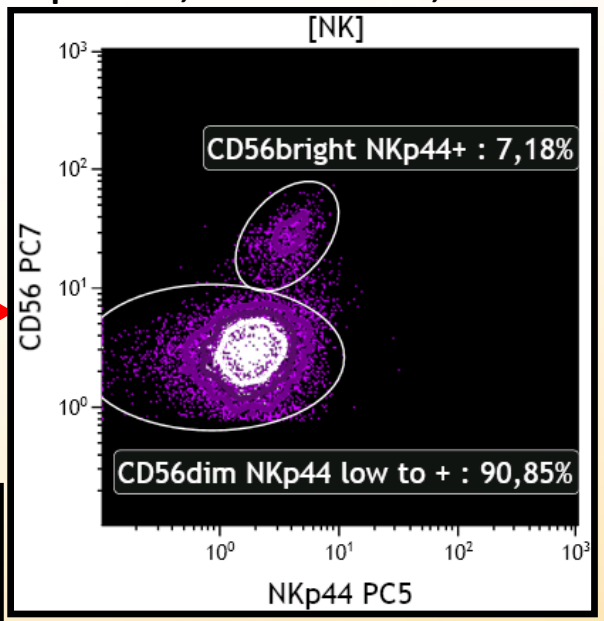


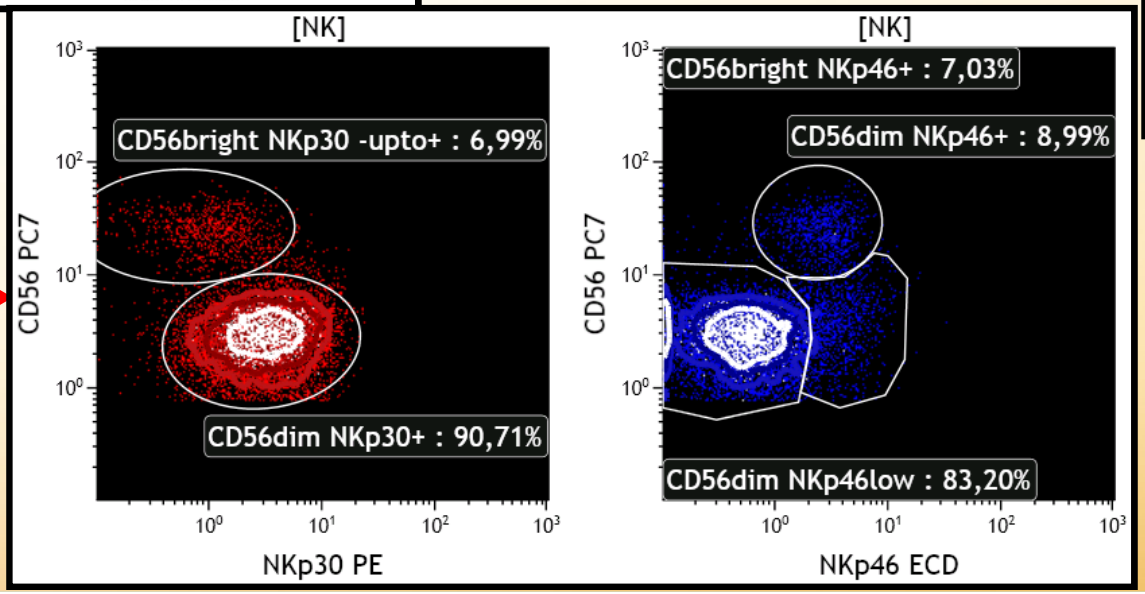
Taken from Llera et al, *J Biol Chem*, 2001,276:7312-19

3.Results : NCR in NK cell population



For NCR evaluation, we made a 9 colors tube mix with anti CD4 KO, anti TCR Vα24 FITC, anti NKp30 PE, anti NKp46 ECD, anti NKp44 PC5, anti CD56 PC7, anti CD3 APC, anti CD16 AA700 and anti CD8 AA750





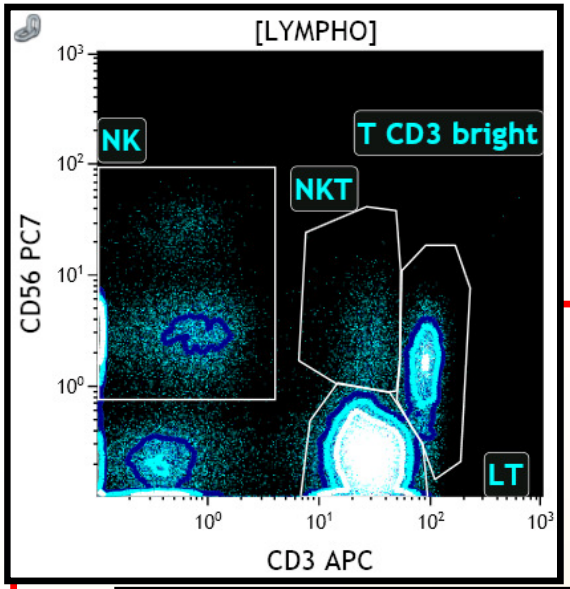
All NK cells were NKp^{+}, interestingly the CD56^{dim} subset was the most NKp30^{+}, and the less NKp44^{+}.

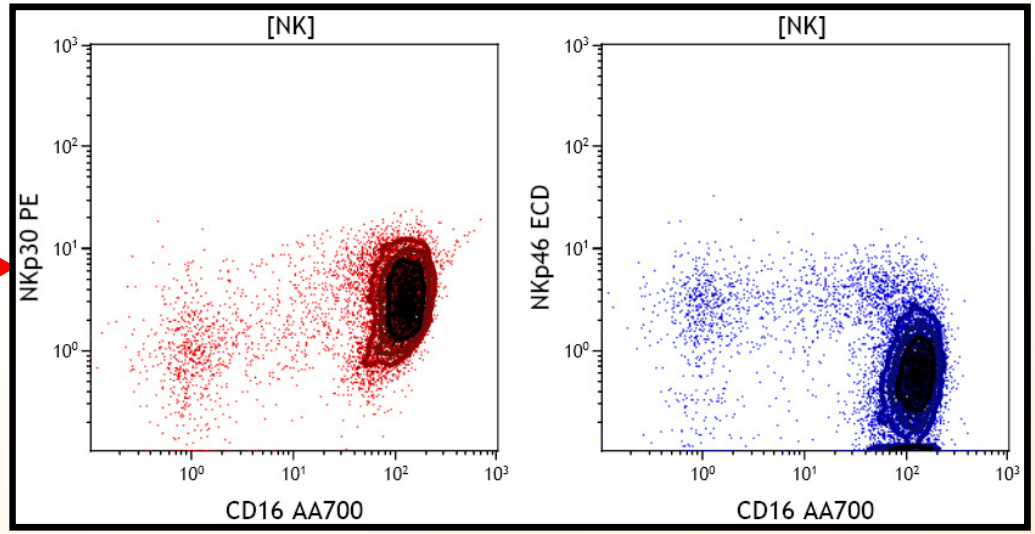
For the CD56^{bright} subset, it was the opposite situation.

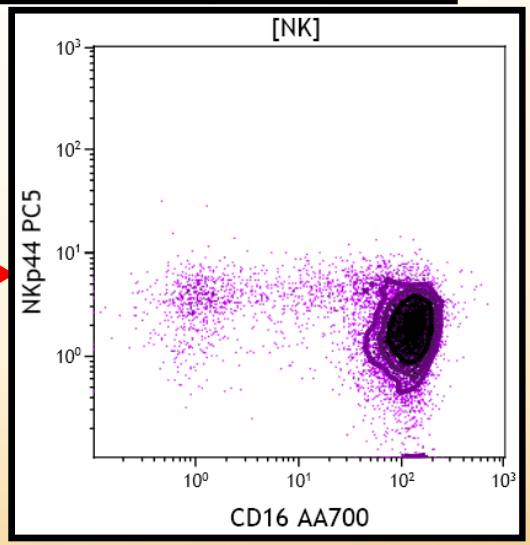
Nkp46 was dimly expressed on the majority of the NK

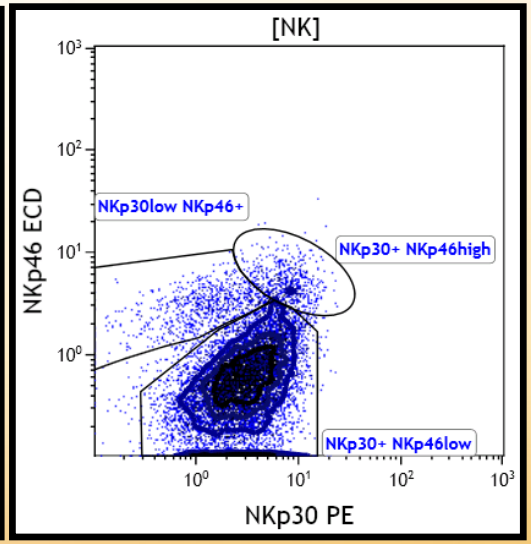
Functional studies might explained if these differences were relevant

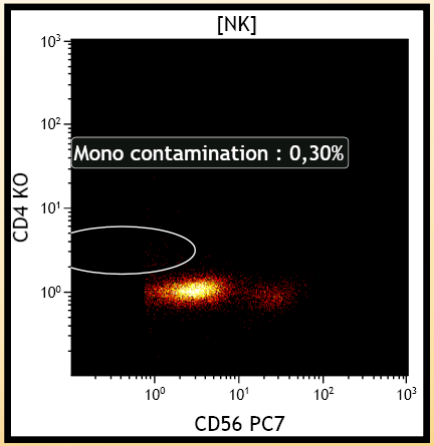
3.Results : NCR in NK cell population







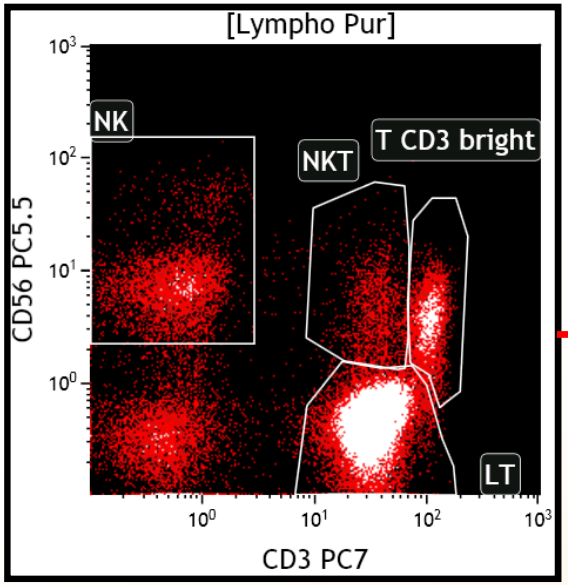


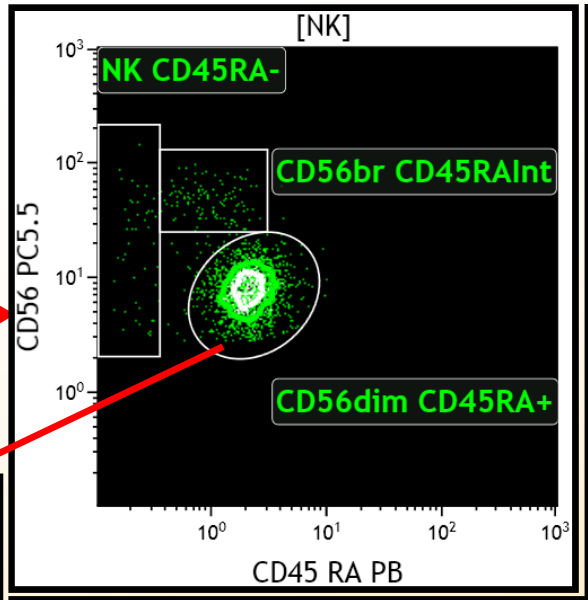


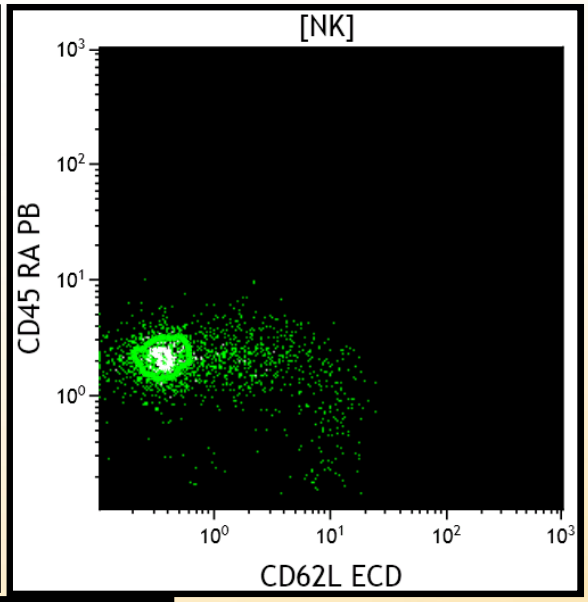
A CD4 vs CD56 plot, gated on lymphocyte, informs about monocytes contamination in the NK gate

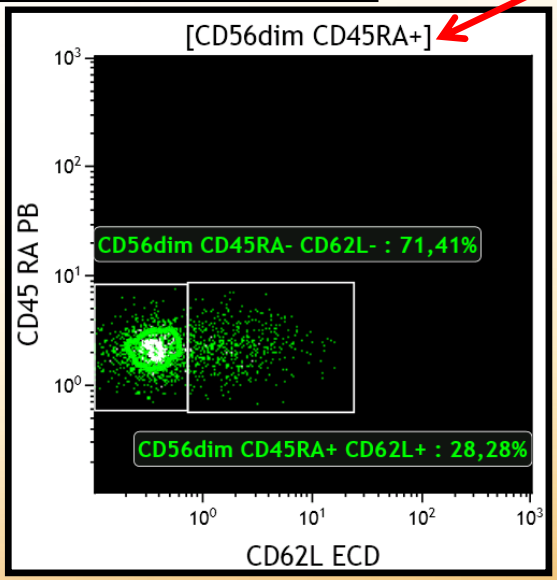
3.Results : NK Populations

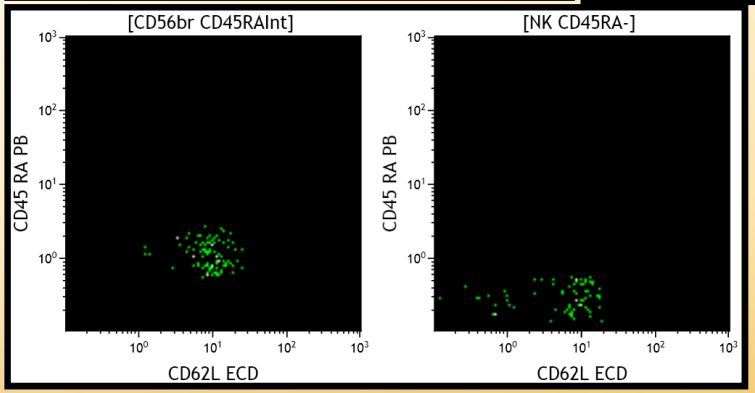
CD45RA and CD62L are well defined for T cells populations (46)
Using this two molecules, we descriminated at least 4 differents subpopulations



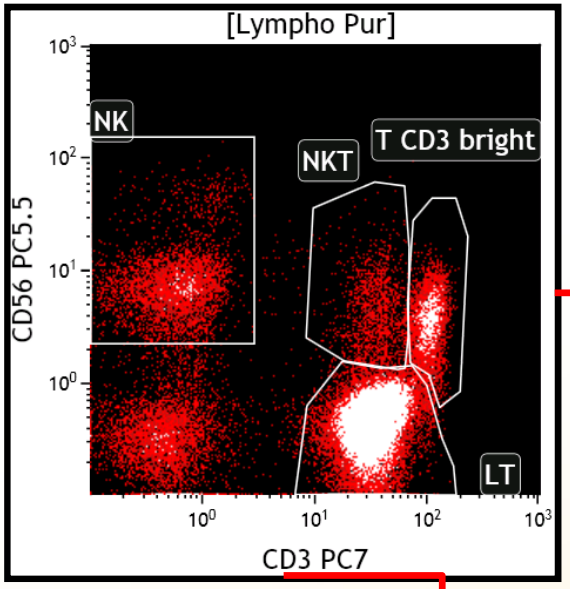




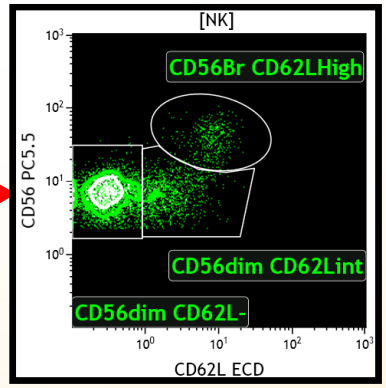


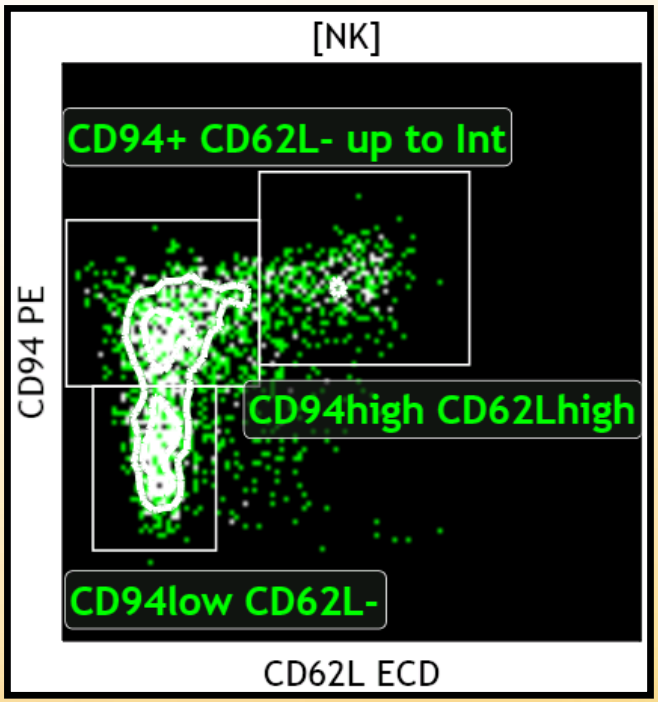


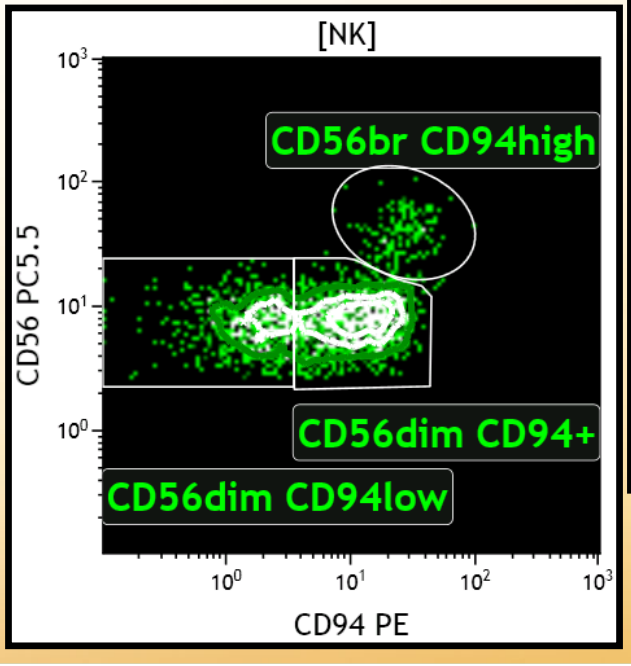
3.Results : NK Populations

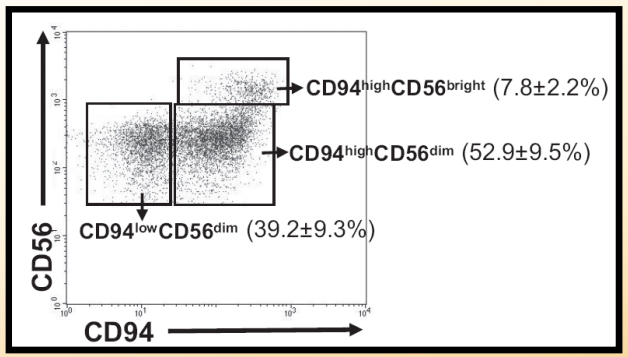


As described [47], CD94 on NK population permits to identify 3 mains populations
Using this molecule couples with CD62L, we descriminated at least 3 differents subpopulations

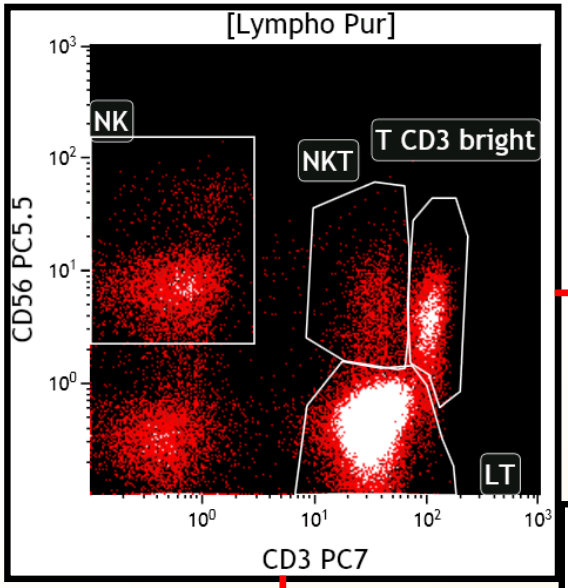




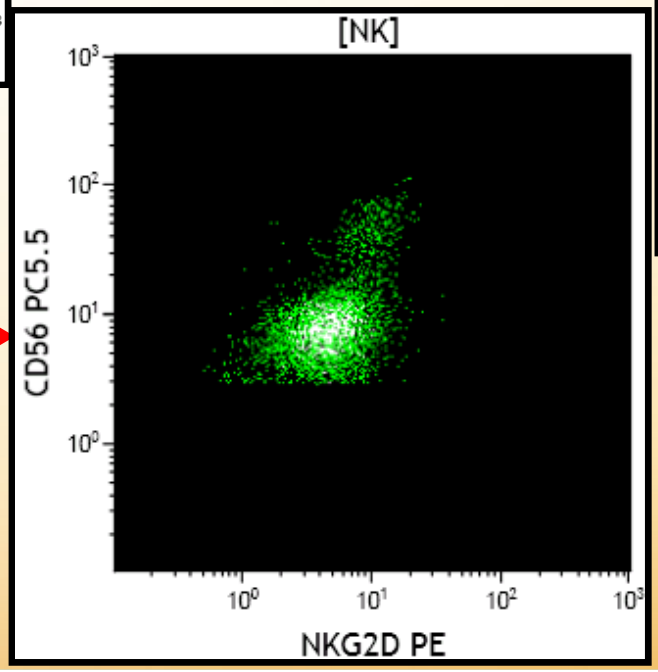


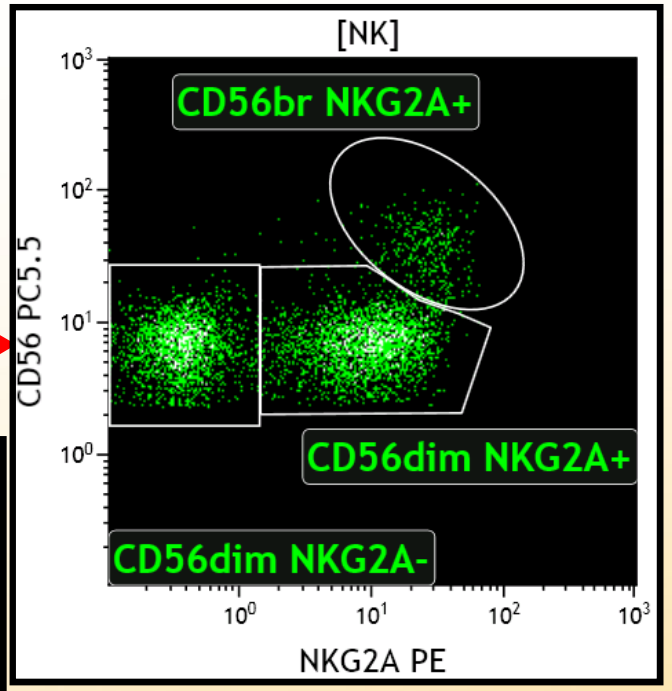


3.Results : NK Populations



NKG2D, NKG2A are important stimulatory and inhibitory receptors that are co expressed with CD94 [10,12]





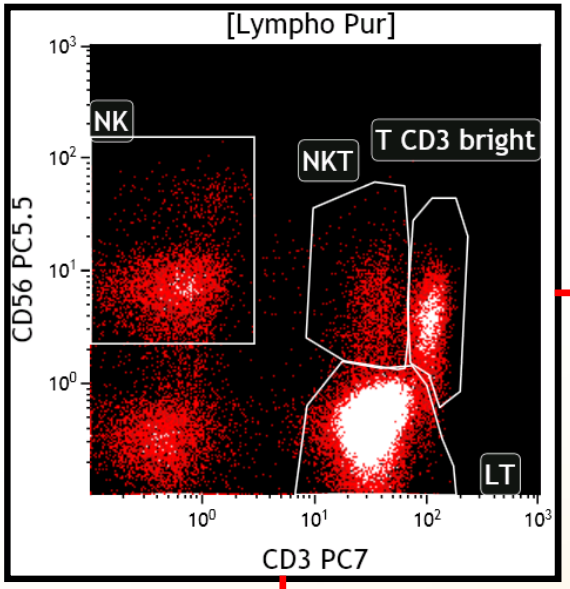
All NK cells were NKG2D$^+$, unlike with NKG2A

There are two populations of NKG2A$^+$

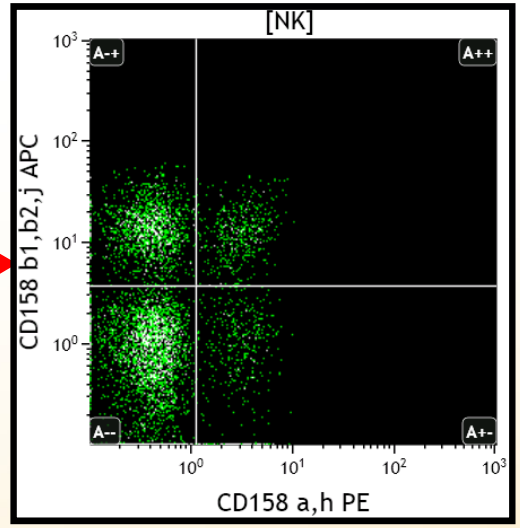
Where as we identified only one NKG2A$^-$ population

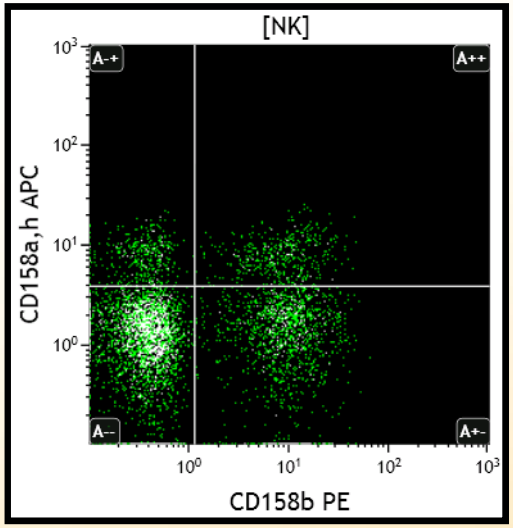
Those cells might be interesting in treatment cancer because they lack the inhibitory receptor for HLA E

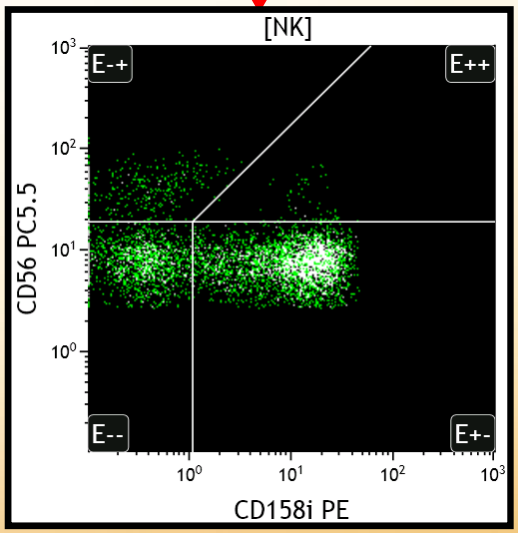
3.Results : NK Populations

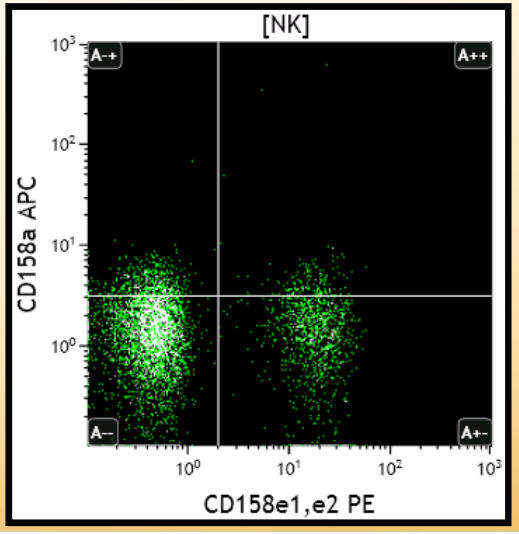


KIRs evaluation is an important point to know which HLA alleles are recognized by killer cells [8]







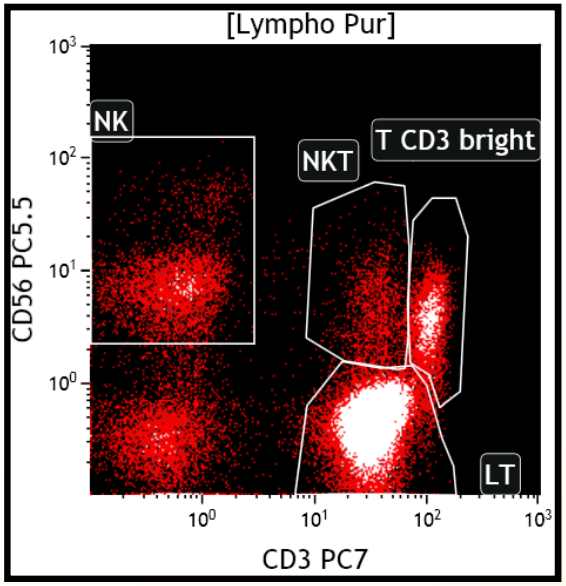


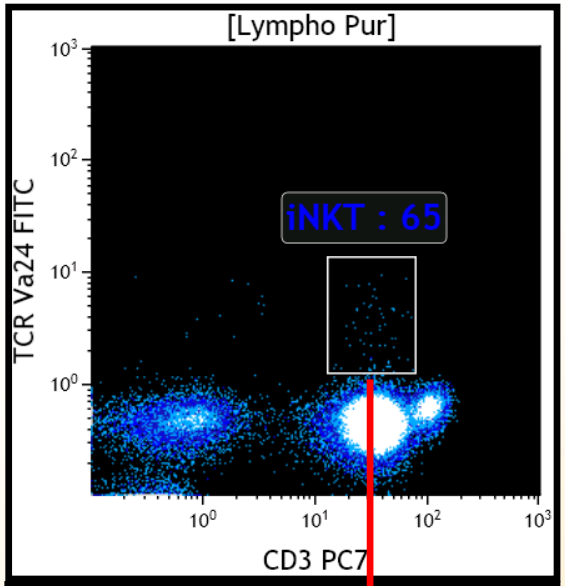
Here we identified NK cell populations which were CD158a$^+$ CD158a,h$^+$, CD158b1,b2,j$^+$, CD158b$^+$, CD158e1,e2$^+$, CD158i$^+$

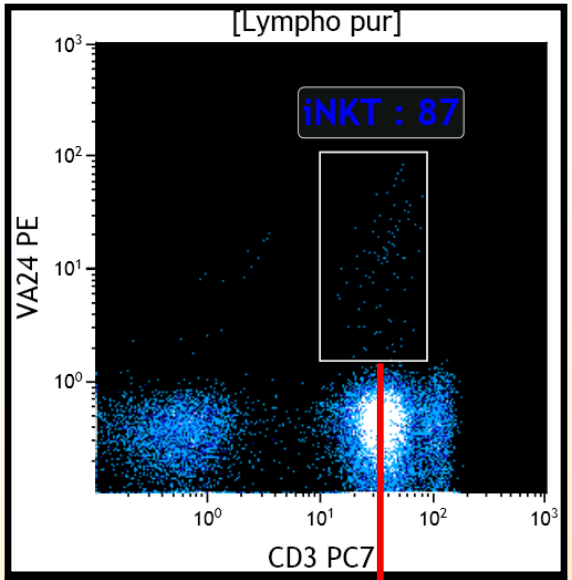
The donor could recognize the HLA-C group 1 and 2 alleles with CD158a,b1,b2,j,h,i KIRs

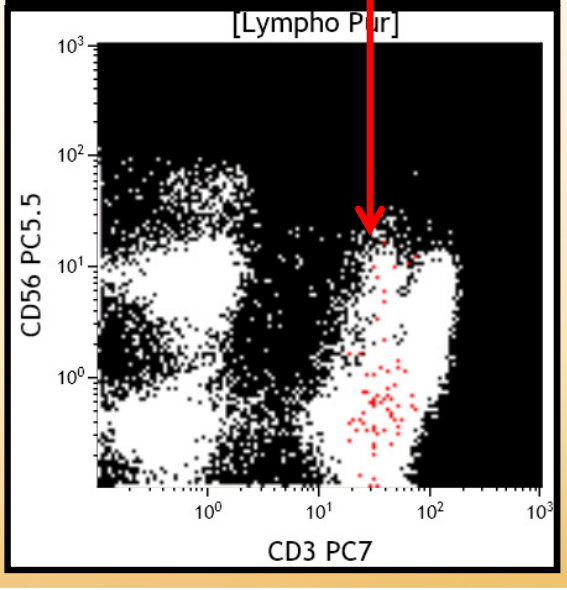
He could also recognize HLA-B and A alleles with CD158e1,e2 KIRs [8]

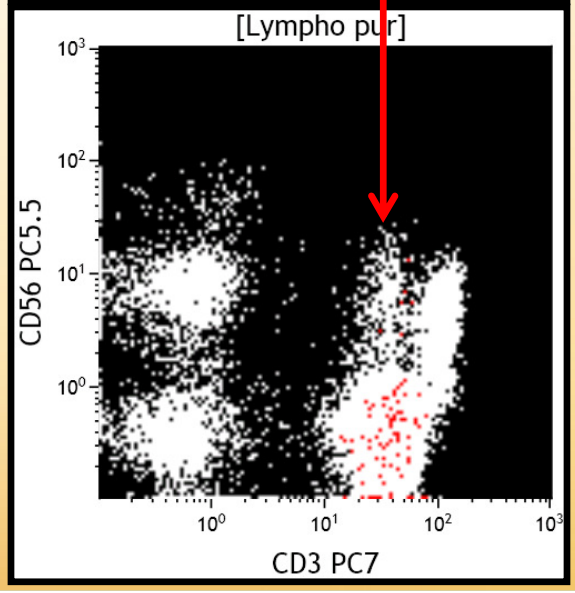
3.Results : NKT Populations











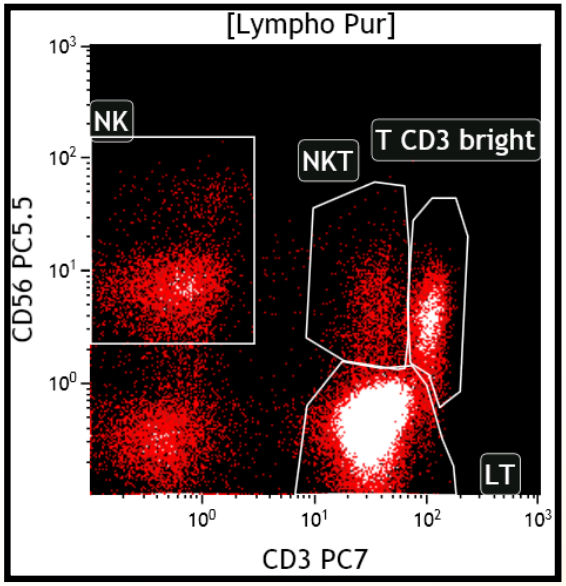
Looking @ TCR Vα24 with FITC or PE coupled antibodies, brings CD3+ CD56+ cells, but CD3+ CD56- cells also

The last aren't type I NKT cells, even there is a lot of work which considers those cells as NKT [31]

These suggests that there are T cells that share the same Vα24 TCR, as type I NKT cells

Use of anti TCR Vβ11 could inform us if those CD3+ CD56- cells really share the same specificity as iNKT cells

3.Results : iNKT Populations



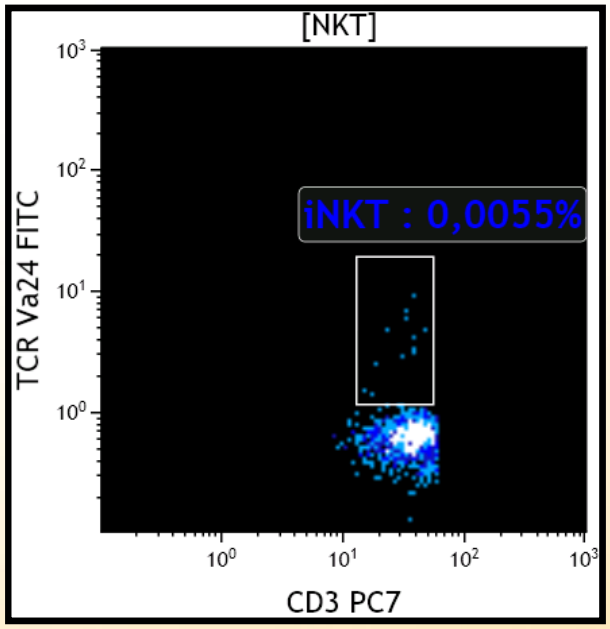
Because of this low frequency, we recommend to make live gate around lymphocytes, and save about 1 million

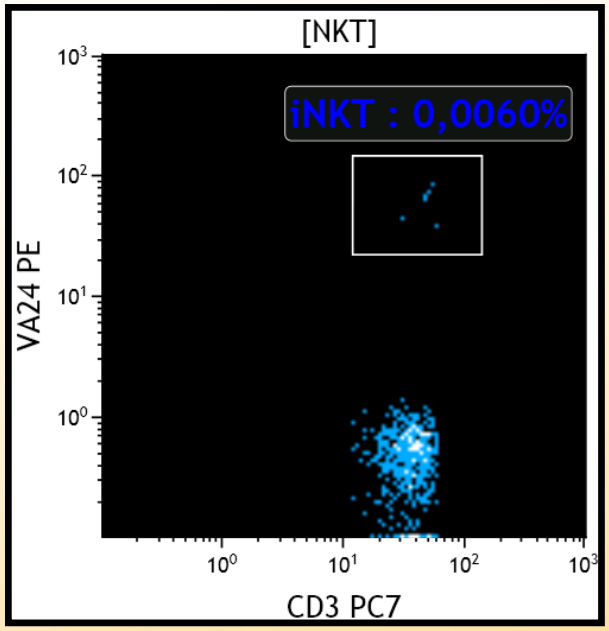
In this way, it may permit to analyze about 2000 iNKT cells

PE coupled antibody offers the best resolution in order to catch iNKT cells

NKT gate permits to really identify the invariant TCR Vα24 CD1d restricted NKT cells

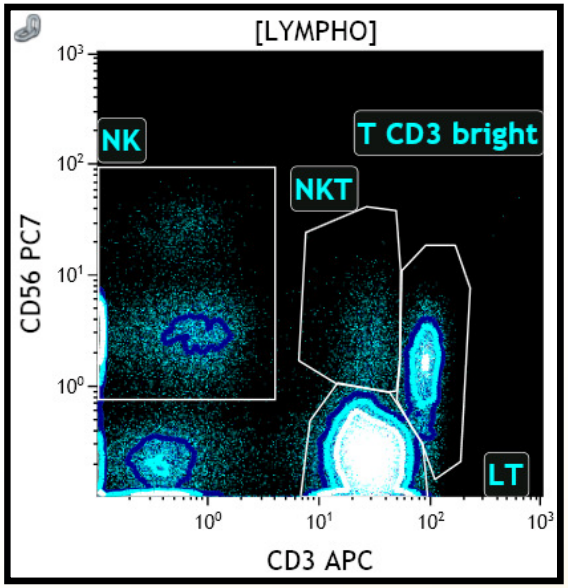
Frequency is about 0,005-0,006% of the total blood cells, about 0,2% of total lymphocytes



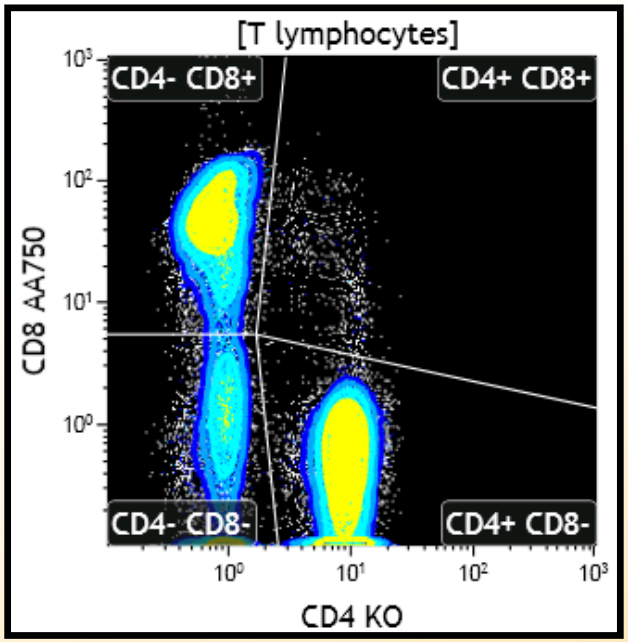


Because we chose to stop acquisition @ 100 000 total cells, it's not very pertinent to analyze so few iNKT cells

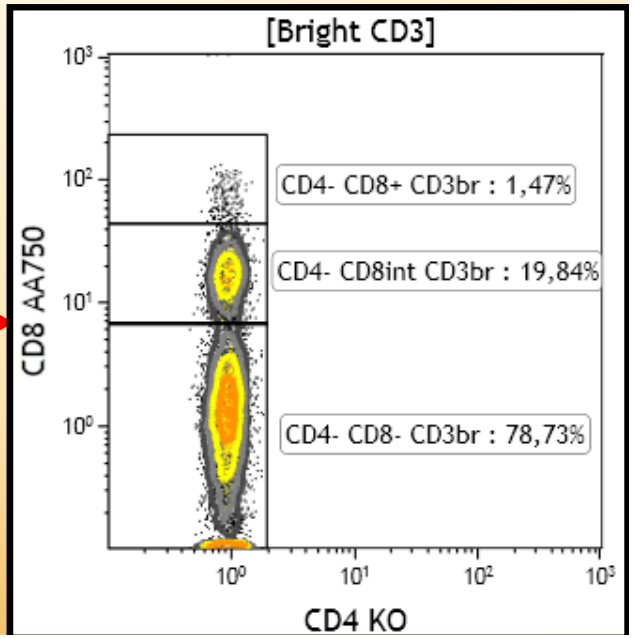
3.Results : CD3^{bright} Populations



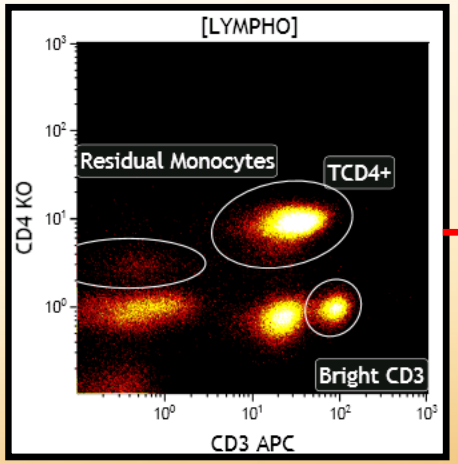
We clearly identify CD3^{bright} which were also CD56^{+} cells, so those cells could also be called NKT cells.
Since they express more CD3, it could be convenient to separate them from the others



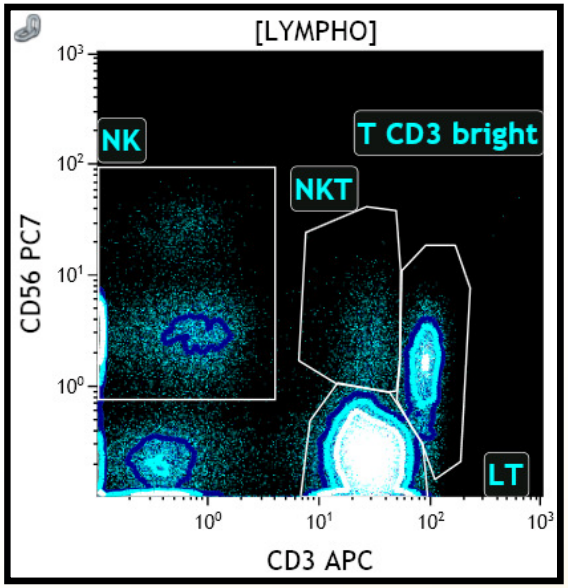
Taking All the T lymphocytes, allow us to make a wonderfull CD8 vs CD4 Dots Plot, and identify single positive populations and a non insignificant double negative population
The majority of the CD3^{bright} was double negative, but we clearly identify CD8^{int} population also

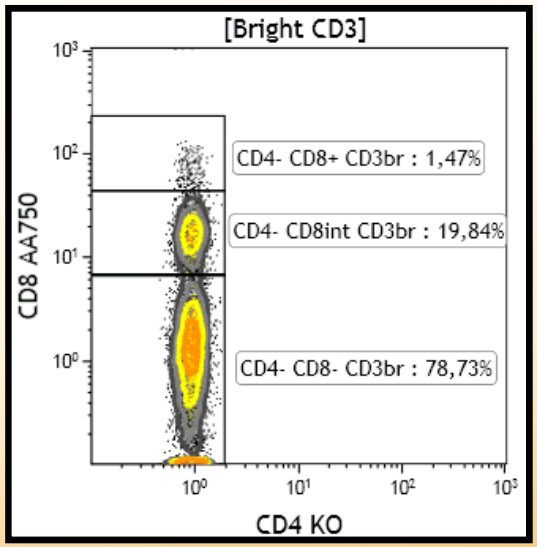


Using the CD3 vs CD4 dot plot, we clearly catch the T CD3^{bright} population

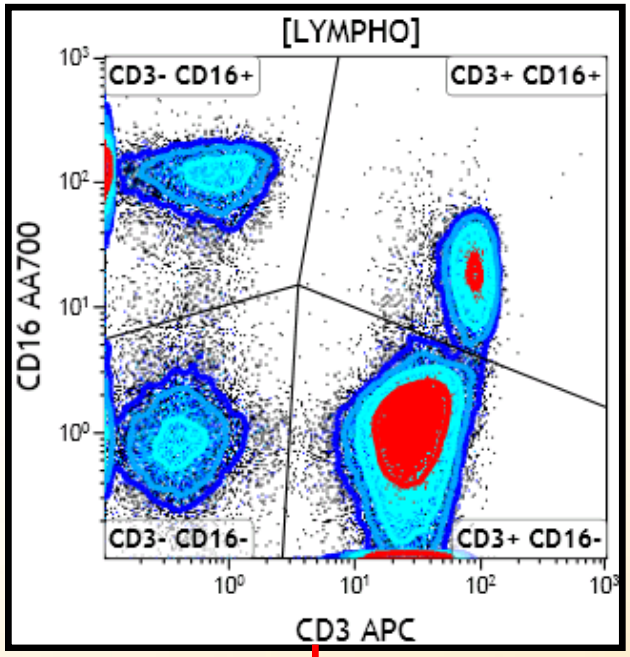


3.Results : CD3^{bright} Populations

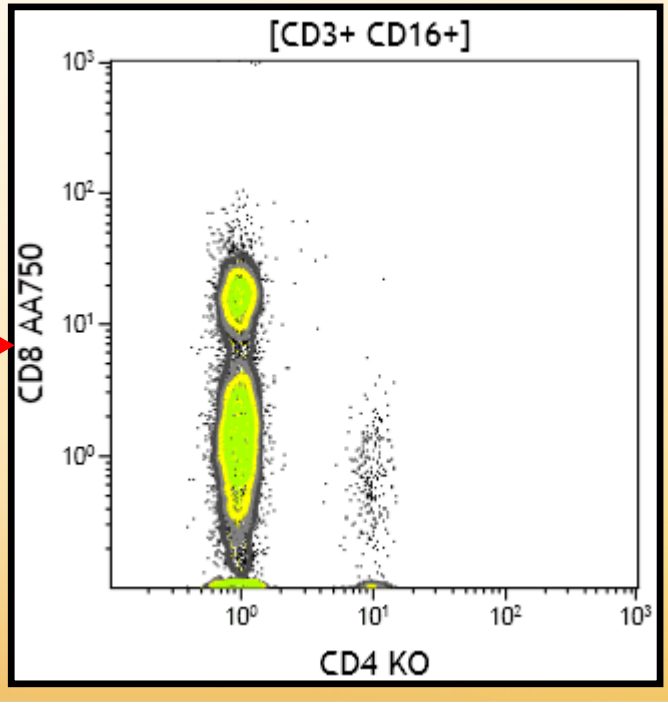




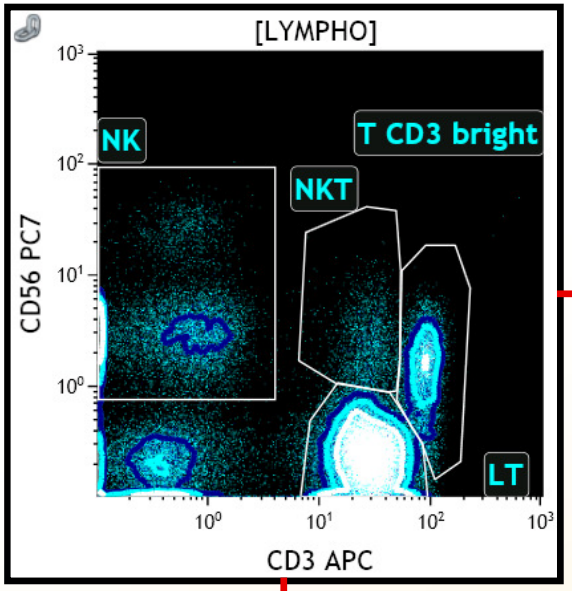
The CD3^{bright} population was clearly CD16^{+}



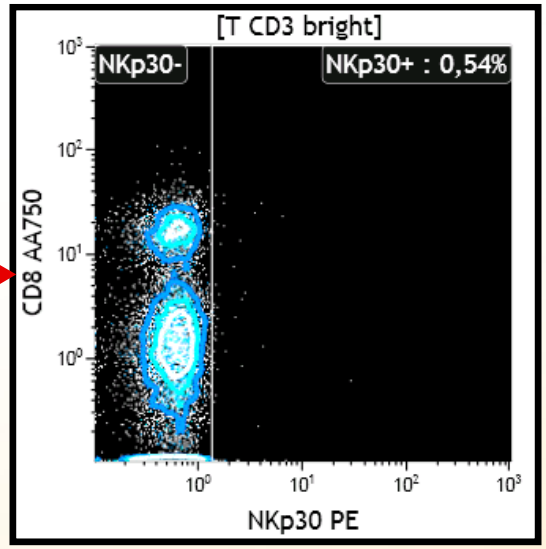
Using the CD3+ CD16+ gate, we found the same CD4/CD8 profile, with a little CD4^{+} contamination

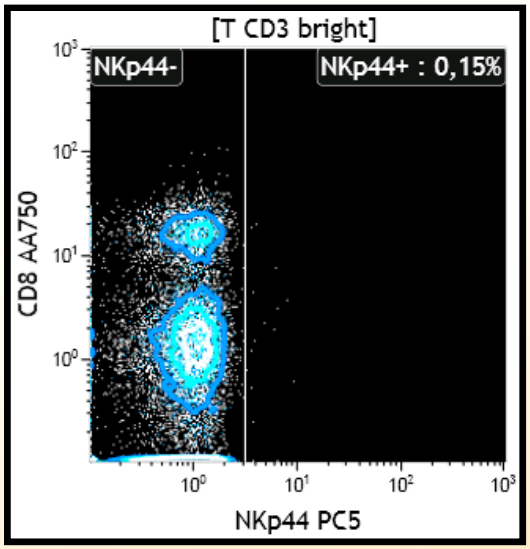


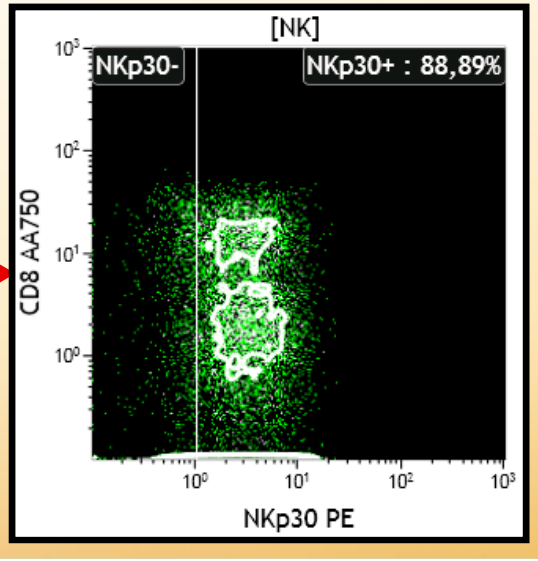
3.Results : CD3^{bright} Populations

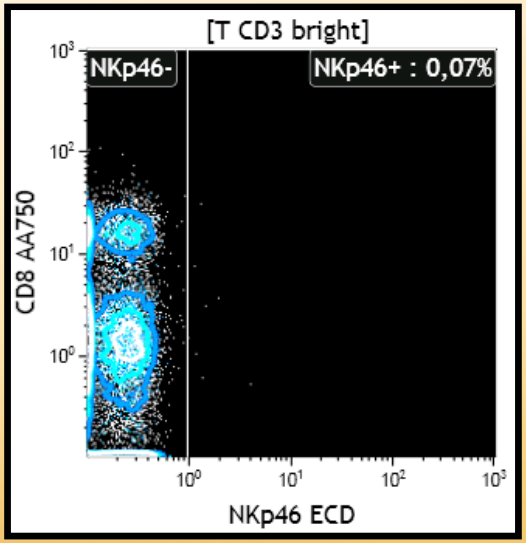


Compare to NK cells, CD3^{bright} population was NKp30, NKp44 and NKp46 $^{-}$ or low



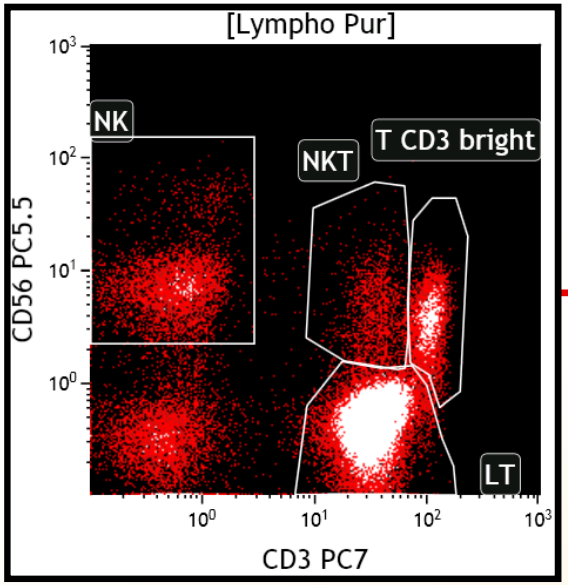


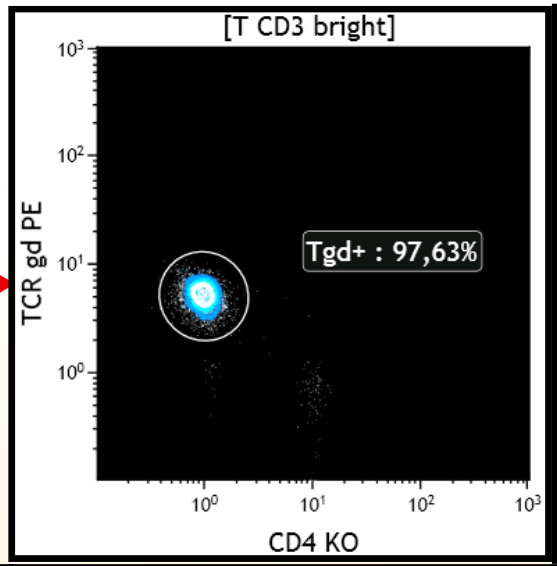


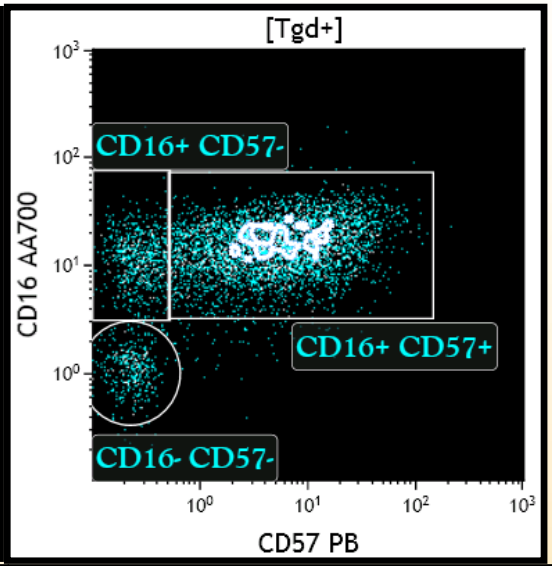


3.Results : CD3^{bright} Populations

CD3^{bright} population express Yδ T Cell Receptor

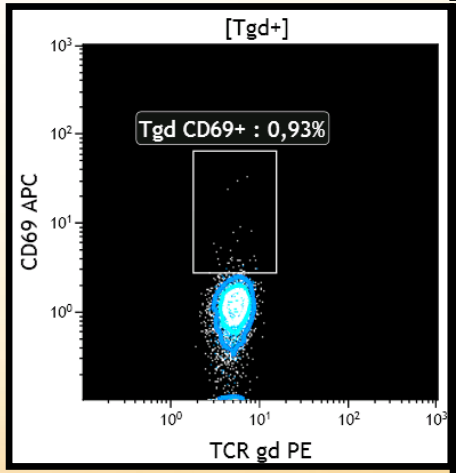


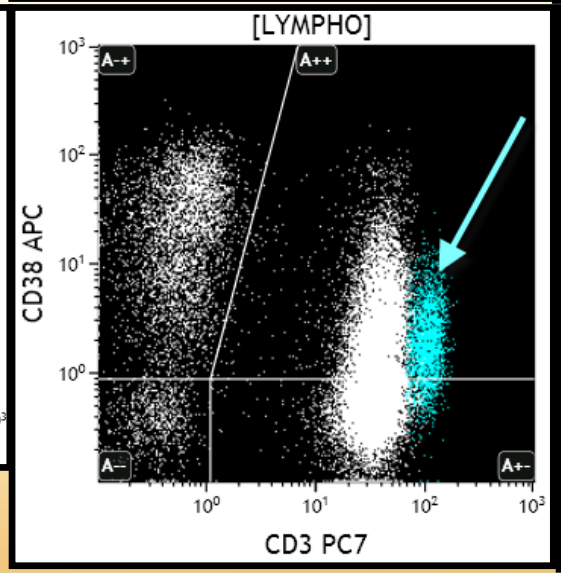


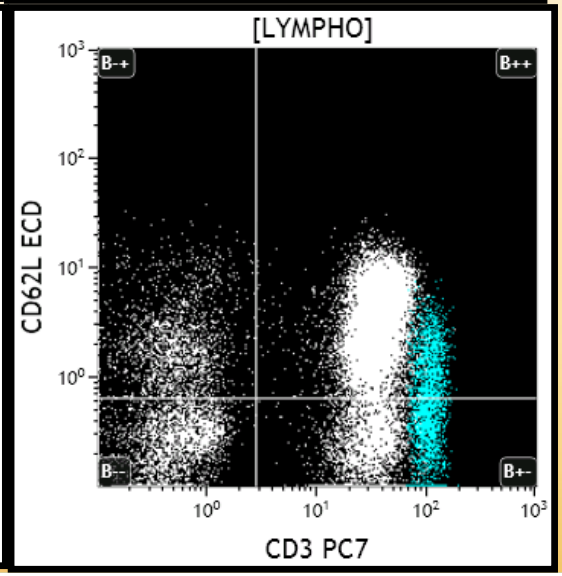


Yδ T Cell could be divided in three subpopulations toward their CD16 and CD57 expression

This population express the CD38 and partially CD62L, but not the CD69

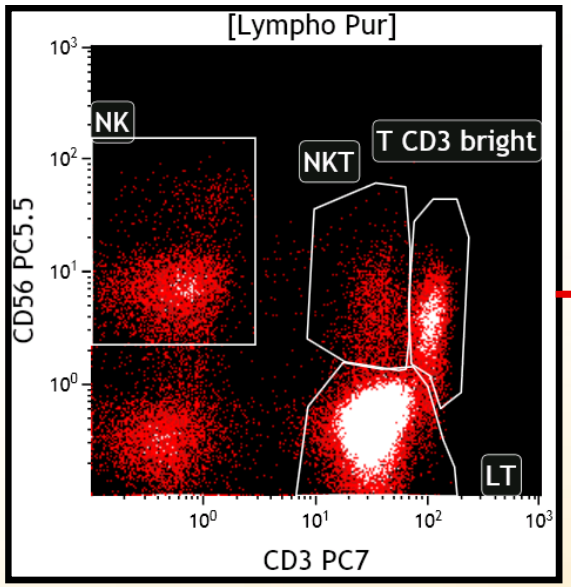


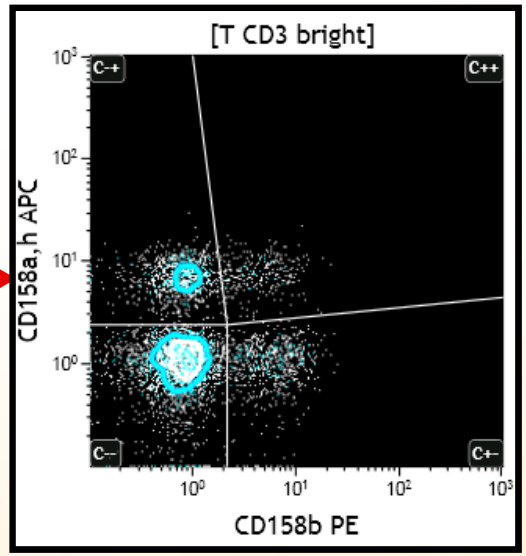


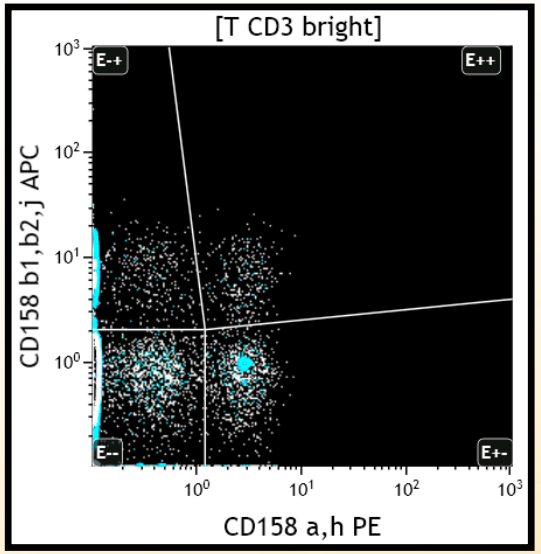


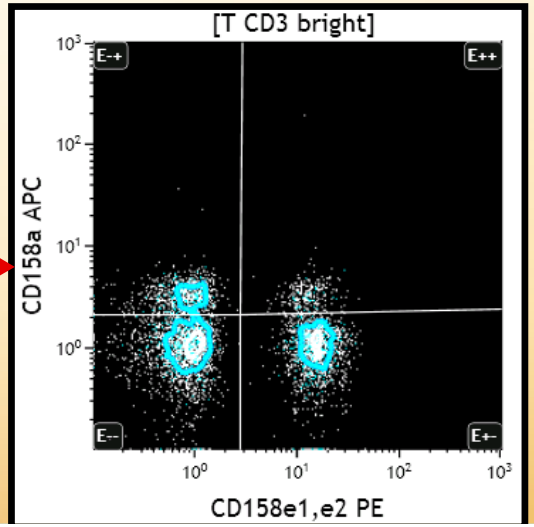
3.Results : CD3^{bright} Populations

It has just been demontrated that CD3^{bright} population express Yδ T Cell Receptor







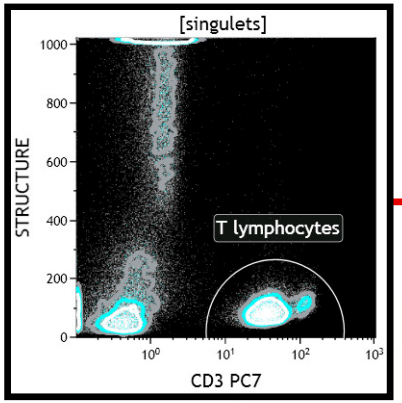


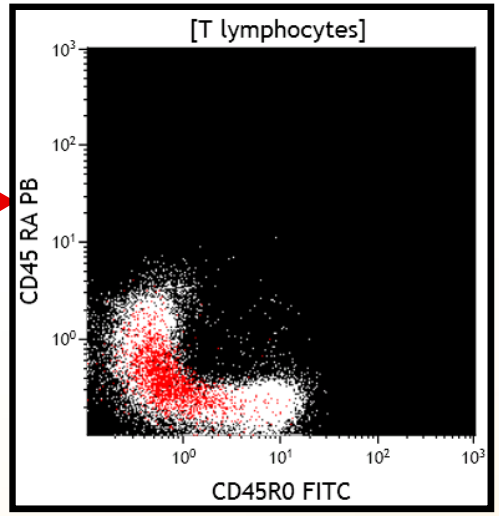
But those cells were trully KIR^{+} also They express all the NK KIR expressed

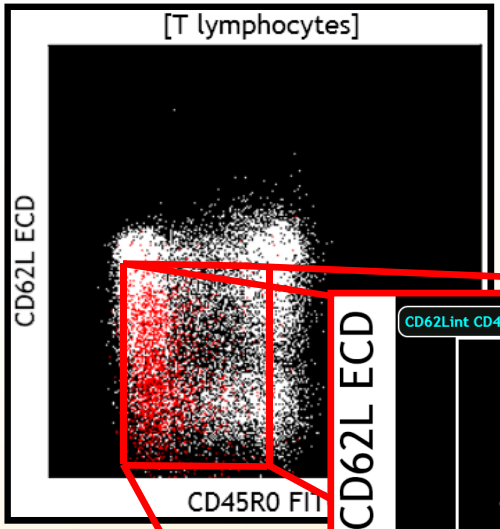
That's a very original point, that those Yδ T Cells express KIRs

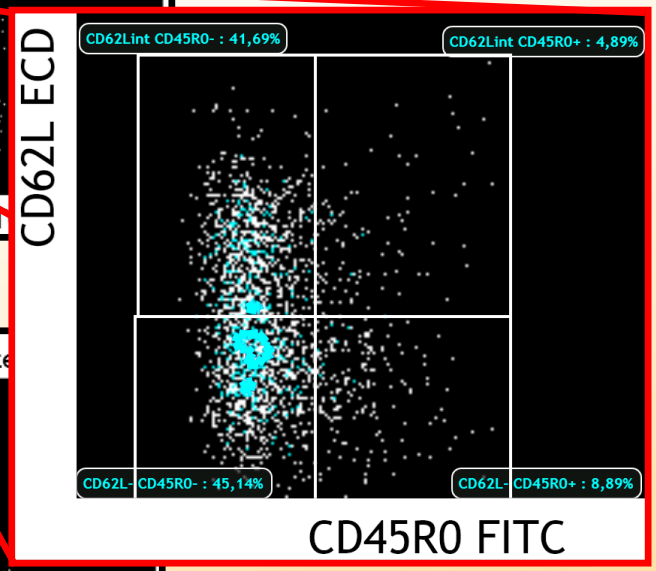
Since they express T cells, and NK cells markers, this Yδ T Cells population could be called NKT cells also

3.Results : CD3^{bright} Populations









Gates colors :

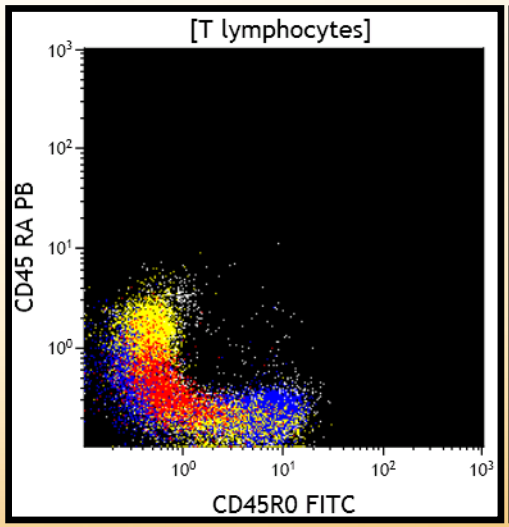
T CD3 bright

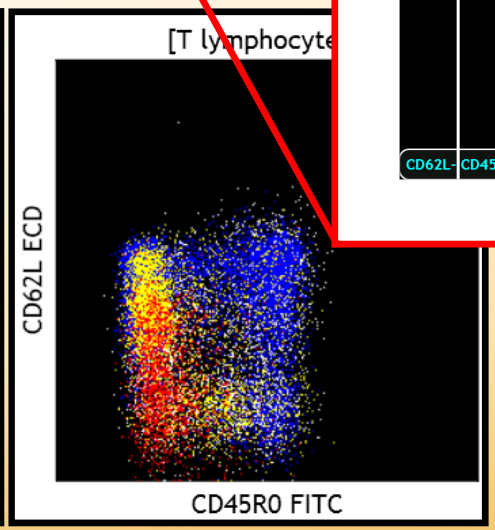
TCD8

TCD4

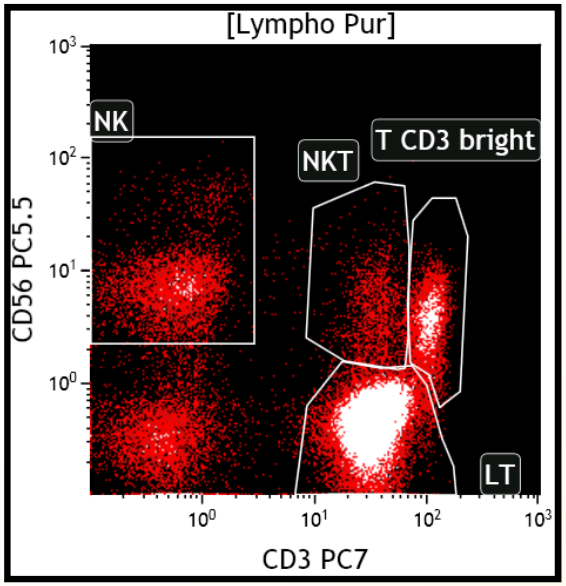
Yδ T cells were CD45RA and R0$^{neg\ or\ low}$ compare to CD4 and CD8 T cells populations

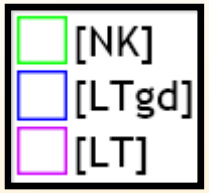
With CD62L marker, we could identify four differents populations

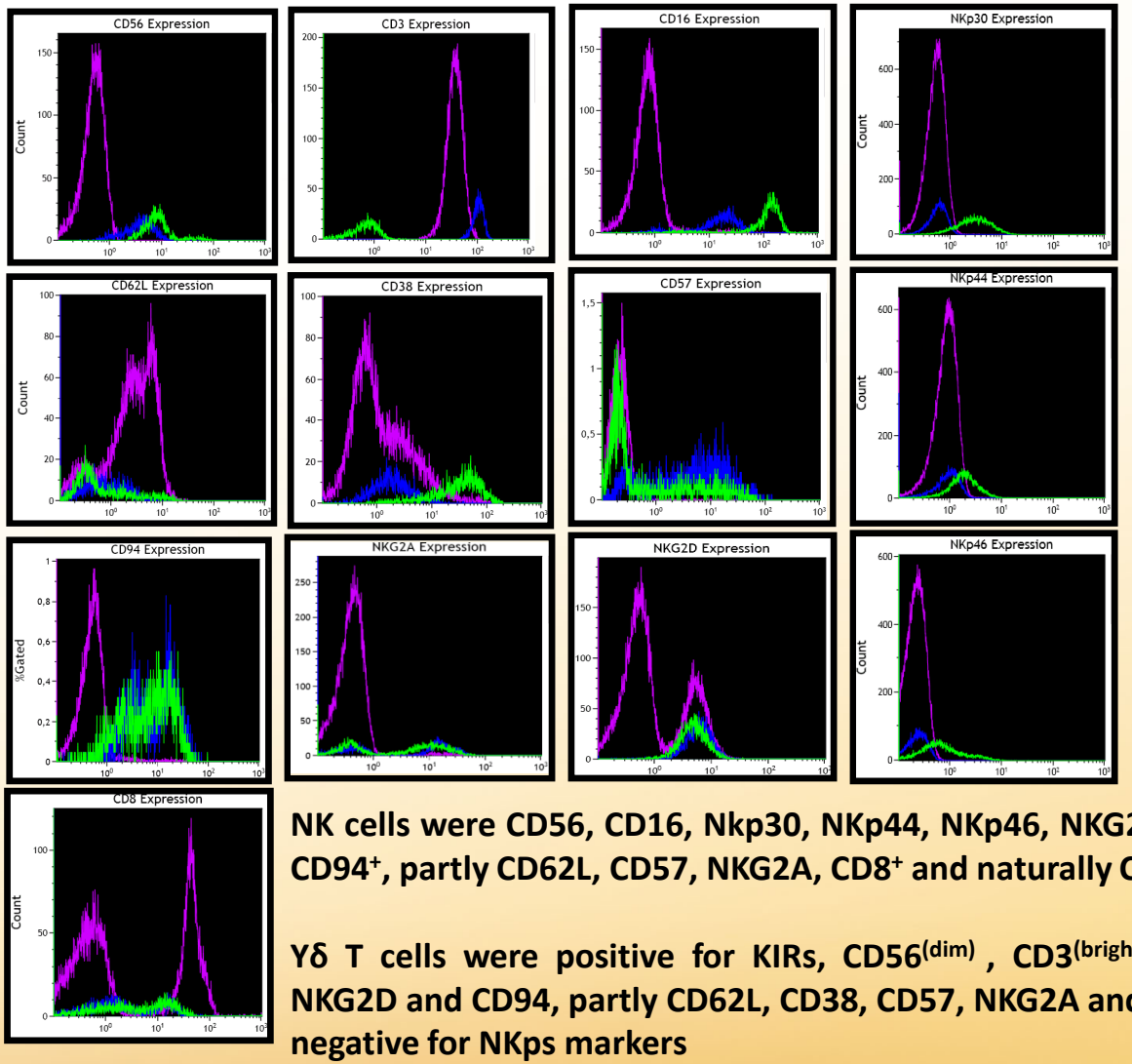




4.Overlaying results



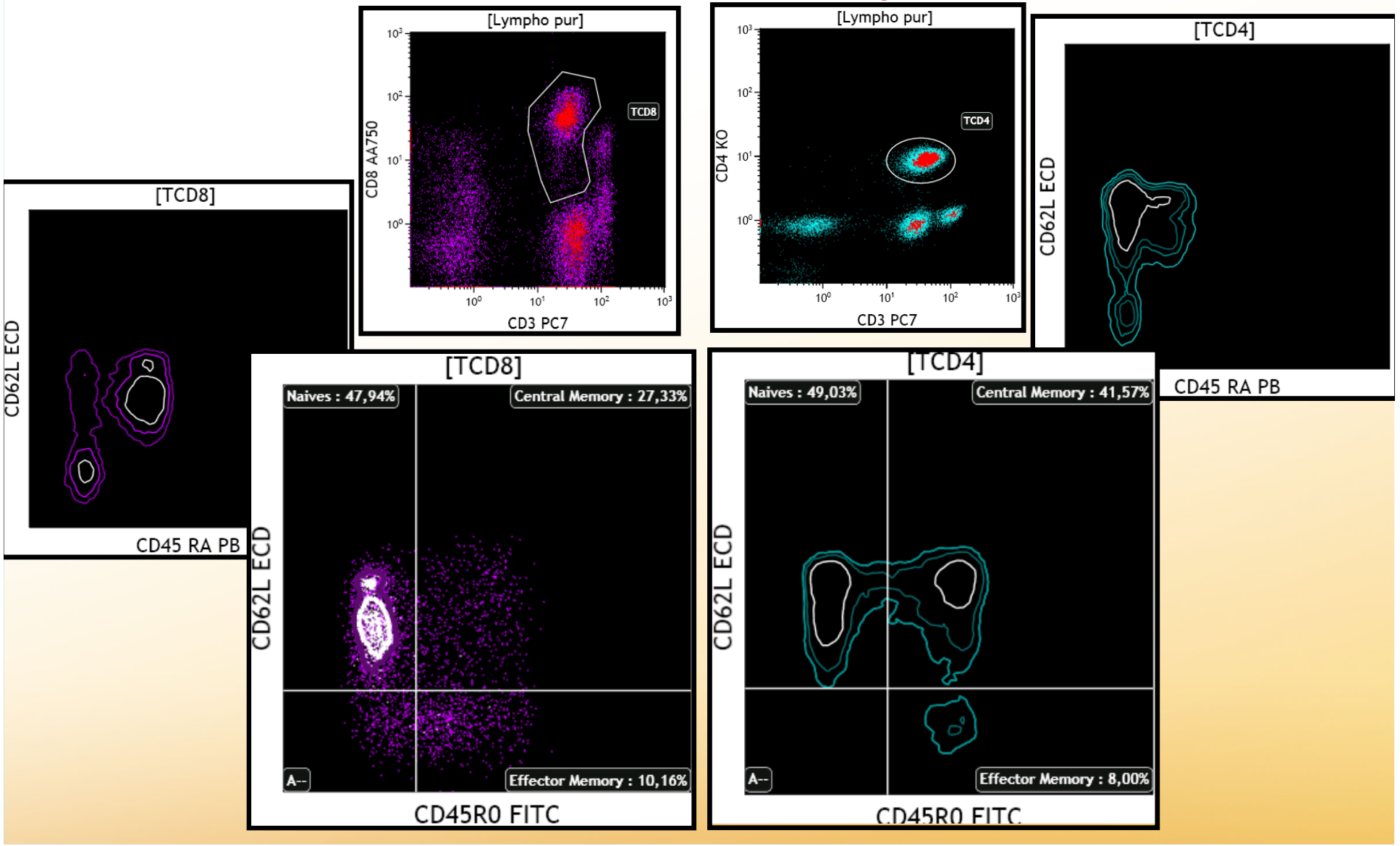




NK cells were CD56, CD16, Nkp30, NKp44, NKp46, NKG2D, CD38, CD94^{+}, partly CD62L, CD57, NKG2A, CD8^{+} and naturally CD3^{-}

Yδ T cells were positive for KIRs, CD56$^{(dim)}$, CD3$^{(bright)}$, CD16, NKG2D and CD94, partly CD62L, CD38, CD57, NKG2A and CD8 and negative for NKps markers

5.Results : T cells naive and memory status



6.Conclusion

- Our 10 colors combinations allow us to clearly identify NK, iNKT, T cells and T $CD3^{bright}$ Yδ cells
- Those combinations, could be very usefull to sort all the populations identified here, and studying them functionnally
- Real iNKT must be confirmed with the use of anti TCR Vα24 + anti TCR Vβ11, and with a longer acquisition
- The specificity of TCR Vα24 $CD3^{+}$ $CD56^{-}$ could be complemented with the use of anti TCR Vβ11
- We clearly see all the NK cells markers, such as NKp, KIRs, NKG2D, NKG2A, CD57, CD94
- Use of anti-CD62L in ECD, could provide a good way to analyze and study the 3 differents NK cells populations, that present different functions
- KIR phenotyping could be easy and very important in KIR mismatch grafting of NK cells
- Yδ T cells really share TCR and KIR, sorting those cells would be of interest in order to realize functional study, or in order to make adoptive transfer in mice

- This work could be a very usefull tool for laboratory working on NK, iNKT, Yδ cells, since it could provide pertinent antibodies/dyes combinations on Navios/Gallios FlowCytometers

7.References

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