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Variability of CD3 membrane expression and T cell activation capacity

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Abstract:

$\alpha\beta$ T cells have a wide distribution of their CD3 membrane density. The aim of this paper was to evaluate the significance of the CD3 differential expression on T cell subsets. Analysis was performed on healthy donors and renal transplant patients by flowcytometry. The results obtained are :

- ❖ CD3 expression was widely distributed (CV = 38.3 ± 3.1 to $43 \pm 2.3\%$).
- ❖ The CD4, CD8, CD45 and forward scatter were similarly distributed.
- ❖ The diversity of CD3 expression was directly related to the clonotypes: $\gamma 9$, non $\gamma 9$ from $\gamma\delta$ T cells and V β clonotype from $\alpha\beta$ T cells (e.g.: V β 3FITC 7980 ± 1628 V β 8PE: V β 20-FITC 11768 ± 1510).
- ❖ Using a computer simulation, we could confirm differential kinetics of T cell activation according to the initial parameters. Finally, *in vitro* activation was significantly higher on V β 8 and V β 9 (high CD3) compared to V β 2 and V β 3 (low CD3, $P=0.040$ to 0.0003).

In conclusion: T cells have highly heterogeneous CD3 expression, possibly predetermined and with clear functional significance.

Keywords:

T cells ; CD3 ; membrane density ; activation kinetics

Abbreviations :

APC	Artificial presenting Cell.
Cy	Cyanine
EDTA	EthyleneDiamineTetraAcetic Acid
FCM	Flow cytometry
FITC	Fluorescein IsoThioCyanate
MdFCS	Median Forward Scatter
MdFI	median fluorescence intensity
MdFCS	Median Forward Scatter
PE	Phyco-Erythrin
TCR	T cell receptor

I. Introduction

During immunization, the T cell receptor (TCR) bind specifically to the peptide-MHC complex if their avidity is appropriate [1-6]. The binding is strengthened by coreceptors, such as, CD4 or CD8 [7-9]. Signal transduction is carried through CD3 that is physically associated to TCR [10]. The CD3-TCR complex is then internalized inducing a significant decrease of membrane CD3/TCR expression and partial activation leads to non responsiveness status until the membrane level of CD3 is restored. [11-14]. A minimal number of TCR/CD3 engagement is required for efficient induction of T cell activation [15-17]. If the number of p-MHC is restricted, serial engagement of TCR are possible [18-20]. Further TCR/CD3 complexes need to be recruited from outside the synapse through surface transfer but this has to be completed during a short lag time [16, 21-23] to be efficient. Activation induce IL-2 production and membrane expression of the γ chain of its receptor (CD25; [24]). IL2 binds to the receptor, induces signal and is internalized for subsequent steps of T cell activation. Membrane CD25 expression is correlated with T cell activation [25].

Experimental data have demonstrated that a number of CD3-TCR is crucial for T cell reactivity. It is thought that the starting membrane density is similar between individual T cells. These processes have been mimicked by computational models [26-29] and compared with published data using well described peptides [6]. IL2-IL2R complex is rapidly produced up to a peak (zenith) before exponential decline. Testing peptides with different avidity have shown that the delay and height of the simulation peak directly reflects of the cell capacity for energy or proliferation [26].

It has been recently shown that CD3 membrane density could be heterogeneously distributed on T cell subsets with a bimodal distribution. Indeed, $\gamma\delta$ T cells do express higher levels of CD3 than $\alpha\beta$ T cells [30-33]. But CD3^{bright} T cells do not include all $\gamma\delta$ T cells. Peripheral $\gamma\delta$ T cells have restricted diversity. In healthy donors, the $\gamma 9$ clonotype is almost exclusively present in blood though in other circumstances, different $\gamma\delta$ isotypes are present in blood [34-36] and express lower CD3 levels. Looking more closely, $\alpha\beta$ T cells themselves appear to have a wide distribution of their CD3 membrane density and their size. This should theoretically influence their capacity to respond [26].

The aim of this paper was to approach the significance of the differential expression of CD3 on $\alpha\beta$ T cells observed in healthy donors and immuno-compromised patients.

I.1. Patients and methods

Lymphocyte analysis was performed on samples from 97 healthy adult blood donors and 56 consecutive samples from renal transplant patients, (time from graft 1-15 years). For regulatory reasons, Blood donor sample could only be tested the day after withdrawal, whereas patient samples were tested fresh, within 6 hours of withdrawal. Functional tests were performed on 10 fresh samples from patients from the cardiology department without immune defect (patients with non severe vascular diseases).

Lymphocytes were immunolabeled in routine practice using a combination of: CD3-Fluorescein IsoThiocyanate (FITC, IgG1, clone sk7), CD16-Phycoerythrin (PE, IgG1, Clone B73.1), CD56-PE (IgG1, Clone NCAM 16.2), CD45-Peridin Chlorophyl Protein (PerCP, IgG1, clone 2D1), CD4-PE-Cyanin7 (PE-Cy7, IgG1, clone SK3), CD19-Allophycocyanin (APC, IgG1, clone SJ25C1), and CD8-APC-Cyanin7 (APC-Cy7, IgG1, clone SK1). An alternative labelling was used for $\gamma\delta$ T cell analysis using: CD3-APC-Cyanine 7 (clone sk7), all from BD Biosciences (San Jose, CA) and CD8 α -APC (clone SFC121thyD3), CD4-PE-Cy7 (clone SFC112T4D11), CD8 β PE-Cy5 (clone 2ST8.5H7) and even TCR γ/δ FITC (clone Immu 510) or TCR $\gamma 9$ (Immu 360) all from Beckman-Coulter; Fullerton, CA.

The clonotypes of TCR V beta chain were identified using pairs of conjugated monoclonal antibodies to: V β 1 (clone BL37.2) PE and V β 2 (clone MPB2D5) FITC, V β 3 (clone CH92) FITC and V β 4 (clone WJF24) PE, V β 5.1 (clone IMM157) FITC and V β 5.3 (clone 3D11) PE, V β 5.2

(clone 36213) FITC and V β 8 (8.1 and 8.2; clone 56C5) PE, V β 7 (clone ZOE) FITC and V β 9 (clone FIN9) PE, V β 11 (clone C21) PE and V β 12 (clone VER2.32.1) FITC, V β 13.1 (clone IMMU222) PE and V β 13.6 (clone JU-74) FITC, V β 14 (clone CAS1.1.3) PE and V β 16 (clone TAMAYA 1.2) FITC, V β 17 (clone E17.5F3) FITC and V β 18 (clone BA62) PE, V β 20 (clone ELL 1.4) FITC and V β 22 (clone IMMU 546) PE, V β 21.3 (clone IG125) FITC and V β 23 (clone AF23) PE (all purchased from Beckman-Coulter). All associated with CD3-APC-Cy7, CD8 α -APC, CD4-PE-Cy7, CD8 β PE-Cy5.

For the activation test, fresh density gradient isolated mononuclear cells (PBMC) were incubated for 30 h with CD3/CD28 coated beads (Dyna; Norway) at approximately equal number according to manufacturer instructions. After incubation, cells from the same well were washed and labeled with three combinations of representative clonotype: V β 1 PE/V β 20 FITC; V β 2 FITC/V β 8 PE and V β 3 FITC/V β 9 PE, associated with CD3-APC-Cy 7, CD8 α -APC, CD4-PE-Cy7, CD25 PE-Cy5 (Clone MA251; BD Biosciences).

The absolute membrane density of receptors was evaluated by indirect labeling, using commercially available QuiFiKit[®] (Dako Denmark) according to the manufacturer instructions.

The immunolabeling procedures were performed on 100 μ L of EthyleneDiamineTetraAcetic Acid (EDTA) anti-coagulated fresh peripheral blood, incubated with 10 to 20 μ L of antibody, gently mixed and incubated for 20 min at room temperature in the dark. The lysis and fixation were obtained by adding 500 μ L of FACSLysing solution[™] (BD Biosciences). Samples were analyzed within 1 h on a FACScanto II (BD Biosciences). For Vbeta labeling, erythrolysis was performed using Versalyse[®] solution (Beckman-Coulter) and samples were washed with 4ml Phosphate buffer before analysis.

Instrument setting were performed on nine tubes (unlabeled, six single labelled, six isotype controls, and the mixed antibodies) using FACS Diva[™] calculation facilities (BD Biosciences) as previously described [37]. Instrument settings were daily checked using standardized (7 color Setup beads). Blood analysing is daily validated using external quality control (Multi-check control; BD Biosciences).

Dot plots were analyzed using FACS Diva[™] (BD Biosciences): CD3 versus side scatter identification. CD4⁺ and CD8⁺ were analyzed on CD3⁺. Vbeta clonotypes were analysed on 30, 000 T cell events, on either CD3+CD4+CD8⁻ or CD3+CD4-CD8⁺ lymphocytes. The $\gamma\delta$ T cells were defined on CD3/ $\gamma\delta$ TCR. γ 9 positive cells were determined on $\gamma\delta$ TCR + T cells. CD4 or CD8 were determined on CD3+/ $\gamma\delta$ TCR negative cells.

The cell size was estimated from forward scatter according to Sloom *et al.* and Symianov *et al.* [38, 39]. In absence of bead that are standardized for the size, we used commercially available beads with different sizes as described by individual manufacturers: Flowcount[®] beads (10 μ m; Beckman-Coulter), QIFIKIT[®] beads (10 μ m, Dako); 7 color setup[®] beads (4 and 6 μ m, BD Bioscience), Trucount[®] beads (4 μ m, BD Bioscience), Rainbow[®] beads (3 - 3.4 μ m, BD Bioscience). The cell sizes were analyzed by electronic microscopy. Briefly, beads were washed in distilled water, treated with cacodylate 0.2M and washed in progressive concentration of ethanol up to 100% before complete desiccation. Samples were then sputtered with gold-palladium coating and analyzed with scanning electron microscope (JEOL 840). More than 25 beads were measured manually on photographs. The reflection index was evaluated at 1.592 for polystyrene beads and 1.456 (Nucleus) and 1.355 (Cytoplasm) for T cell. The size of the nucleus was estimated at 80% of the cell size according to the literature [38]. The bead and cell volumes were calculated from measured FSC peak height according to Mie's theory for spherical multilayer particles optics.

Statistical analysis was performed using, paired Student's *t*-test for comparison of two cell sub-populations from the sample, linear regressions and expressed as median \pm 1SD. Coefficient of variation (CV) were calculated as 1SD/mean.

Because the fluorescence intensity did not always have a normal distribution, we chose to consider the median (MdFI) instead of the mean fluorescence intensity (MFI). MdFI and each sample CV were calculated automatically by the FacsDiva software.

II. Results

II.1 Wide heterogeneity of CD3 expression inside T cell subsets:

In routine blood T cell analysis, we observed a wide distribution of CD3 expression on T cells (Figure 1a). We have confirmed this interindividual cell diversity in 96 healthy donors: the MdFI was 1100 ± 178 on CD8+ T cells meaning a 16.1% coefficient of variation (CV) between donors.

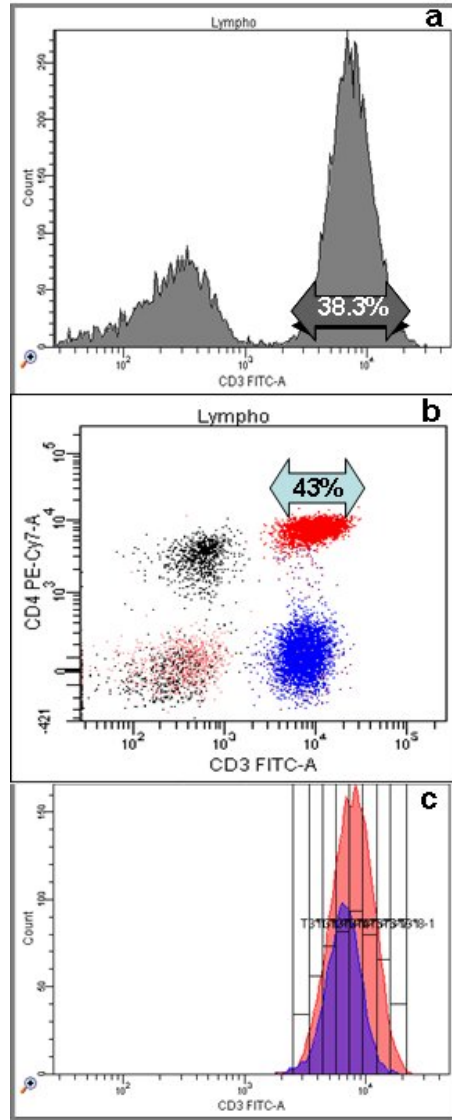


Figure 1: High variability of CD3 FITC fluorescence intensity observed on CD3FITC/CD56 PE/CD4PE-Cy7/CD19APC/CD8APC-PE-Cy7/CD45PerCP routine lymphocyte analysis (one representative case; panel a). CD3 expression was higher on CD4+ T cells compared to CD8 (panels b & c).

The CD3 expression was significantly ($P < 0.0001$) higher on CD4+ T cells ($1,401 \pm 248$) with similar CV (17.7%; Figures 1b and 1c). However, what was more intriguing was the wide variation between cells inside each sample (intraindividual) CV: $38.3 \pm 3.1\%$ for CD8+ T cells and $43 \pm 2.3\%$ for CD4+ T cells (Figure 1b). A similar discrepancy was observed on 56 consecutive patients (intraindividual CV: $32.7 \pm 2.7\%$; on CD8+ and $37.0 \pm 2.3\%$ on CD4+ T

cells). The discrepancy of CD3 expression was confirmed with an alternative anti-body combination that included CD3 APC-Cy7, CD8 APC, CD4 PE-Cy7: CVs = 27.8±6.4% on CD8+ and 34.4±6.7% on CD4+ T cells (n= 40 patients).

II.2. The IntraIndividual Variability was Repeatedly Observed in all T Cell Isotypes:

There is good evidence that CD3 expression differs amongst some T cell subtypes particularly $\alpha\beta$ or $\gamma\delta$ TCRs. We could confirm that CD3 levels were significantly higher on $\gamma 9$ $\gamma\delta$ T cells (MdFI: 17,933 ±3 214 in 40 patients) compared with conventional T cells (paired *t*-test: *P* = 0.0001). Similar to the conventional T cells, the CD3 expression was highly heterogeneous on $\gamma 9$ $\gamma\delta$ T cells (mean of individual CVs: 36.6±8.0% Figure 2a). Conversely, the CD3 expression was not so high on “non- $\gamma 9$ ” $\gamma\delta$ T cells (14,005 ±4 386 compared to $\gamma 9$ $\gamma\delta$ T cells ; *P* = 0.0001) but still significantly higher than on $\alpha\beta$ T cells (*P* =0.0187) and large individual CVs: 36.4±9.5%.

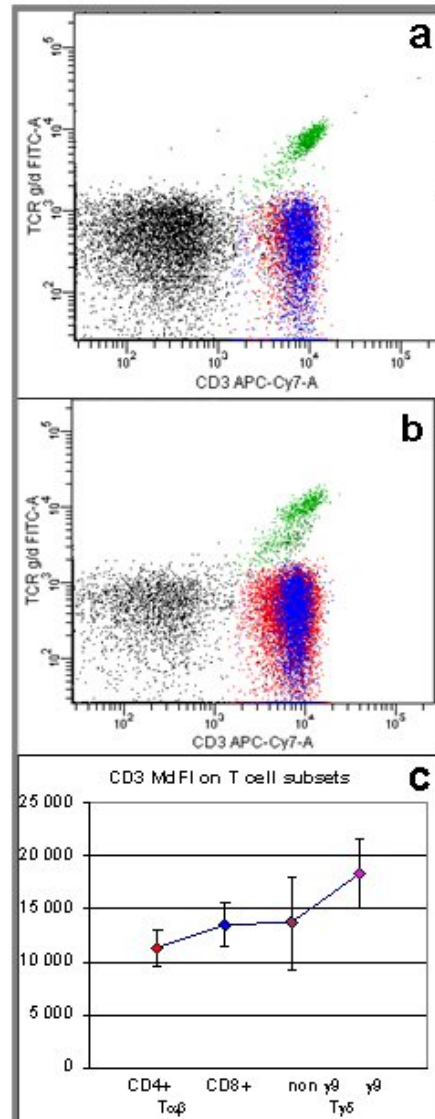


Figure 2: CD3 APC-Cy7, CD8 APC, CD4 PE-Cy7 and $\gamma\delta$ FITC were tested on 40 patients. CD3 expression was widely distributed on classical $\gamma 9$ peripheral $\gamma\delta$ T and higher compared to $\alpha\beta$ (panel a). Some patients had an additional non $\gamma 9$ population of $\gamma\delta$ T cells (panel b) with even wider distribution of CD3. The two $\gamma\delta$ populations had significantly higher CD3 expression compared to CD4+ and CD8+ $\alpha\beta$ T cells (panel c).

In the same experiment, we have compared the levels of CD3 expression on CD4+ and CD8+ T cell after exclusion of $\gamma\delta$ T cells that partially express CD8. Again, we observed that the CD3

APC-Cy7 expression was higher on CD4-PE-Cy7+ (MdFI: 13,535 ±2 020) than on CD8-APC+ αβ T cells (MdFI: 11,435 ±1 717; $P < 0.0001$; Figure 2c). Because possible artifacts could be due to the spectral overlap compensations between the CD8 APC and the CD3 APC-Cy7 signals, we have confirmed the difference of CD3 expression between CD4+ and CD4- αβ T cells in five patients using the same labeling combination except CD8 APC (“fluorescence-minus-One” procedure; results not shown).

II.3. Were There Differences of CD3 Expression Between TCR Vβ Clonotypes?

Because CD3 levels of expression varied between γδ clonotypes, we investigated also differed between αβ clonotypes.

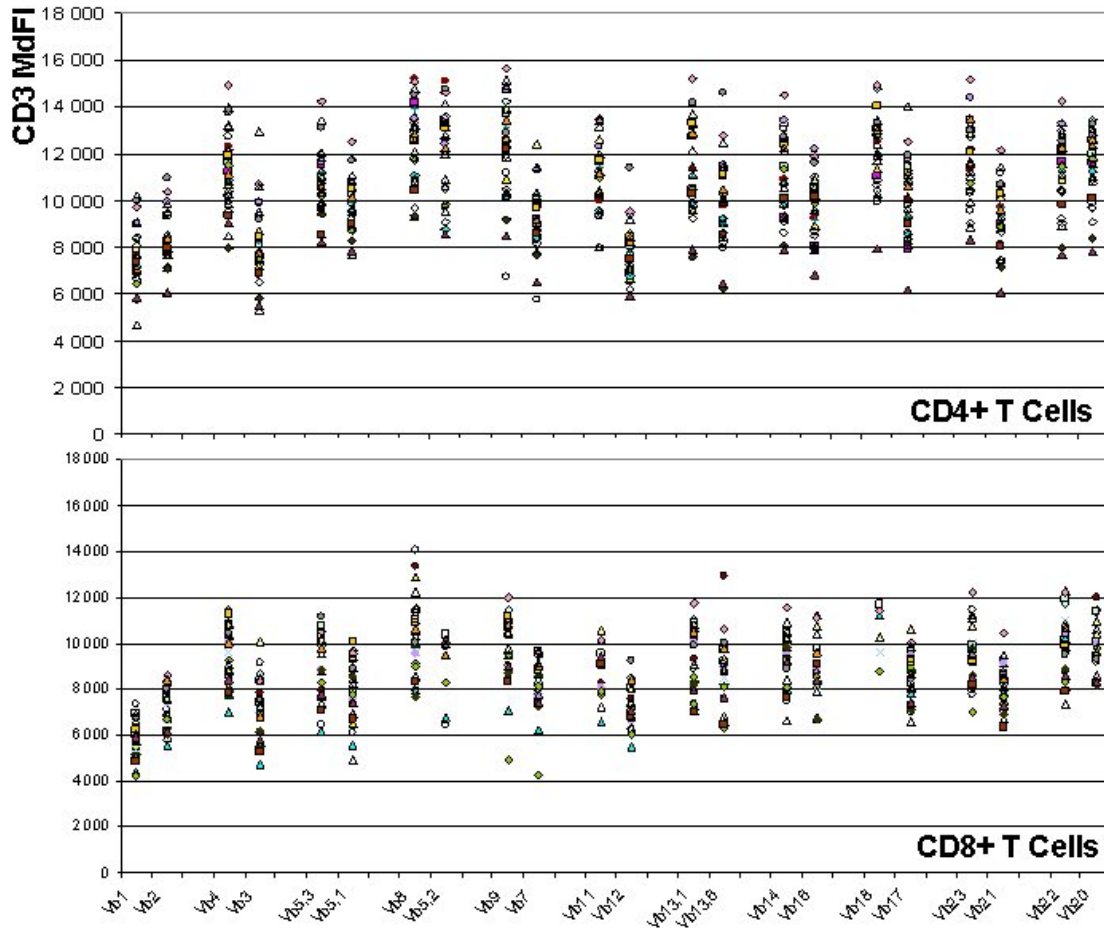


Figure 3: CD3 APC-Cy7, CD8 APC, CD4 PE-Cy7, and Vβ clonotype FITC/PE were tested on 28 healthy donors. CD3 median fluorescence intensity (MdFI) was reproducibly different according to the clonotype of the cells. Values were always higher on CD4+ (upper) than CD8 T cells (lower panel) T cells.

Using CD3 APC-Cy7, CD8 APC, CD4 PE-Cy7, and two anti Vβ clonotypes conjugated with either FITC or PE in 28 healthy blood donors, we observed consistent differences of CD3 expression according to the Vβ clonotype (Figure 3). MdFI were repeatedly very low on some clonotypes (e.g. for CD4+ T cells: Vβ1 PE: 7,366 ±1,236; Vβ2 FITC 8,023 ±1,140, Vβ3 FITC 7,980 ±1,628) and very high (paired t -test: $P < 0.0001$) in few other clonotypes: (e.g.: Vβ8 PE: 12,845 ±1,695; Vβ9 PE 12,435 ±2,093; Vβ20-FITC 11,768 ±1510). In accordance with the first experiments, the CD3 expression was always higher on CD4+ than on CD8+ T cells but the differences between the clonotypes were the same for the CD4+ cells (Figure 3 upper panel) and the CD8+ T cells (Figure 3 lower panel). The differences were repeatedly observed in all patients and were not related to the fluorochrom (PE or FITC) used for the anti-clonotype

conjugates: on clonotypes that were labeled with either FITC or PE, the CD3 was low (e.g. anti V β 1-PE or anti V β 2-FITC) or high (e.g. anti V β 3-FITC or V β 20-PE) (see Figure 3).

II.4. Was the Variability of Expression Restricted to CD3?

If CD3 expression had wide variability in each samples, we also observed high variability of expression of other membrane receptors: CD45: 6,269 \pm 893 (CV = 14.2%) on CD4+ T cells and 7,976 \pm 1,186 (CV = 14.9%) on CD8+ T cells; CD4: 7,159 \pm 755 (CV = 10.5%) on CD4+ T cells and CD8: 12,054 \pm 1,518 (CV = 12.6%) on CD8+ T cells in 56 consecutive patients.

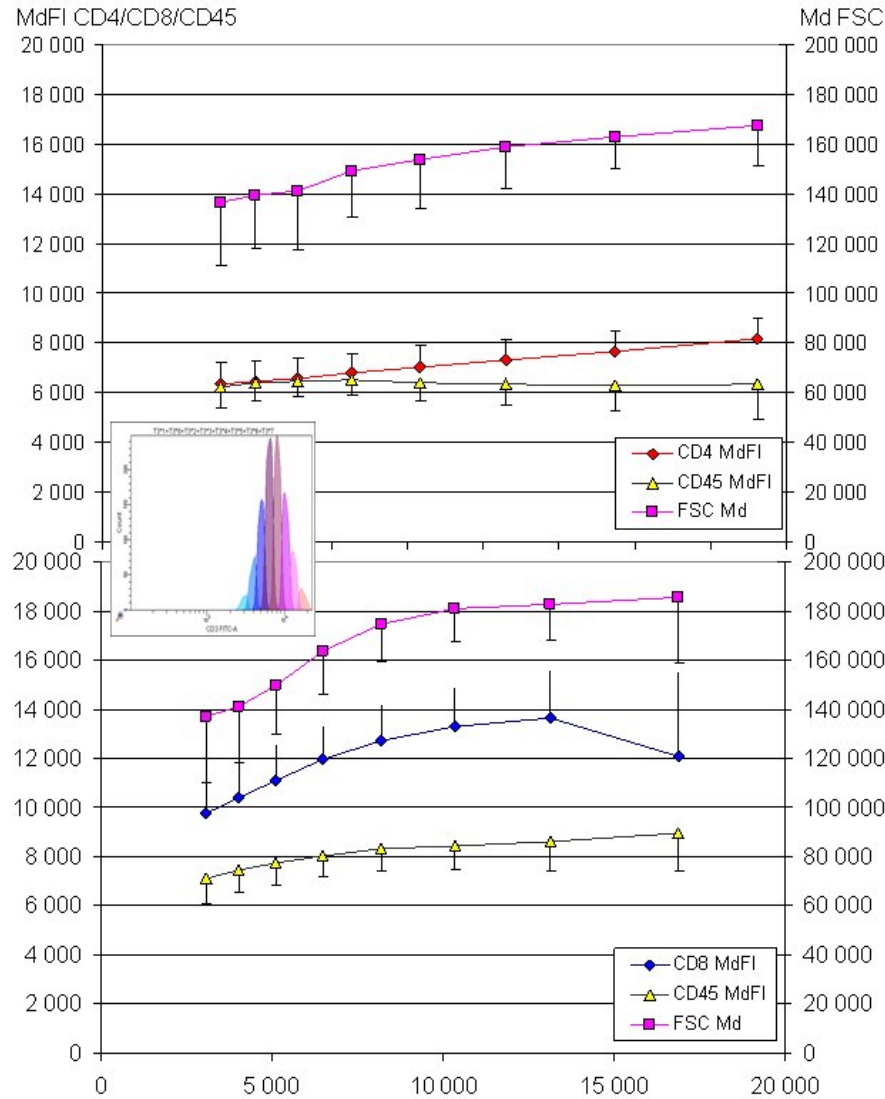


Figure 4: CD3FITC/CD56 PE/CD4PE-Cy7/CD19APC/CD8APC-PE-Cy7/CD45PerCP was analyzed on 56 consecutive patients. The levels of CD4 or CD8; CD45 (left scale) and forward scatter (FSC; right scale) were analysed on CD4+ (upper panel) or CD8+ (lower panel) according to the level of CD3 arbitrarily divided in 8 equidistant sections (insert). CD4 and partly CD8, FSC increased with CD3 expression suggesting a cell size effect.

To compare the level of two receptors, we have arbitrarily divided the T cell population in eight equidistant sections on the logarithmic scale of CD3 fluorescence intensity (Figure 4, insert). We then compared CD4 or CD8 and CD45 levels of expression between these CD3 sections. The CD4 gradually increased with CD3 levels ($P < 0.001$, paired t tests between two consecutive CD3 levels sections). The CD8 also gradually increased in all but the highest

sections ($P < 0.001$). Similarly, the CD45 gradually increased on CD8+ T cells ($P = 0.001$) but only in the first five sections of CD4+ T cells ($P < 0.026$).

II.5. Was the Difference of Expression Due to Size Diversity of the Cells?

Because the levels of expression of all receptors tested were varying in the same way, we wanted to know if it was related to differences in sizes. The median of forward scattering intensity (high of the peak: MdfSC-H) gradually increased with the level of CD3 (Figure 4) on CD4 + T cells (upper panel) and CD8 + T cells (lower panel, $P < 0.0001$ on paired t -test for each sections compared to the previous one with lower CD3 level).

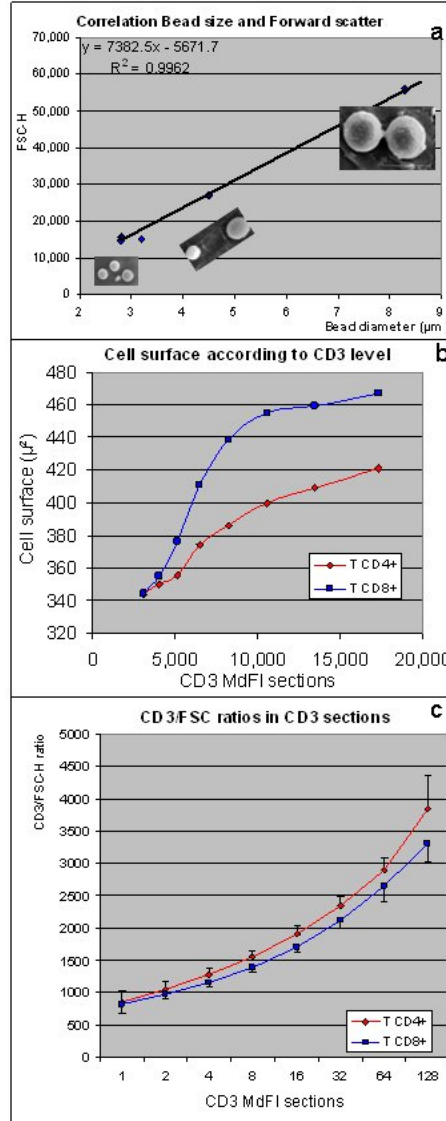


Figure 5: Relationship between forward scatter and particle size a) on beads with different size measured by scanning electronic mMicroscope; b) Cell surface calculated from forward scatter according to the level of CD3 arbitrarily divided in equidistant sections in 56 consecutive patients; c) direct CD3 intensity / FSH ratio on individual cell sizes in the CD3 sections in 14 consecutive patients.

To estimate the cell sizes from the MdfSC, we have first determined the size of some setting beads that could be used as standards, by scanning electronic microscopy (Figure 5a). The bead diameters were: $8.35 \pm 0.32\mu\text{m}$ for the Qifikit® beads (n=34); 3.22 ± 0.089 (n=25) and $4.48 \pm 0.16\mu\text{m}$ for the 7-color setup® beads (n=48); $2.49 \pm 0.89 \mu\text{m}$ for the Trucount® beads (n=37); $8.60 \pm 0.22\mu\text{m}$ for the Flowcount® beads (n=31) and $27.8 \pm 2.8 \mu\text{m}$ for the Rainbow® beads (n=75). We then analyzed their FSC-H (five repeats), using the same settings as the one

used for the cell analysis. We could confirm that the bead MdfSC-H were directly correlated to the bead size in a linear relation) $y = 7382.5x - 5671.7$; $r^2 = 0.996$ (Figure 5).

According to the Mie's theory and reflection index of cells, we could estimate the cell size (diameter, d) with the function: $[FHS-H = 1250 d^2]$ showing that the FSC-H was proportional to the cell surface rather than their diameter.

Accordingly, we have analyzed, the mean cell diameter of cells was 11.1 μm for LyT CD4+ and 11.5 μm for CD8+ T cells in the 56 consecutive samples. Interestingly, the cell sizes progressively increased with levels of CD3 from 10.5 to 11.6 for CD4+ and 10.5 to 12.2 for CD8+ T cell (Figure 5b). In another test, we have considered the CD3 / FSC-H ratio measured individually on each cell during analysis of 14 consecutive the samples. The ratio progressively rose with the level of CD3, from 853 ± 163 to $3,843 \pm 523$ for CD4+ T cell and 829 ± 144 to $3,314 \pm 303$ for CD8+ T cells (Figure 5c) confirming the close relationship between the level of CD3 expression and the cell size.

However, CD3 fluorescence intensities rose more than the cell size (Figure 5b). This suggests that the differences in size were not sufficient to explain the large variability of CD3 expression and larger cells did indeed express high surface density of CD3, especially on CD8+ T cells.

II.6. Could the CD3 Differential Expression Have Functional Consequences?

Because the number of CD3, as part of the TCR complex, is crucial threshold for T cell activation after specific peptide recognition, we investigated whether differences in expression could influence the T cell reactivity. We simulated the kinetic effect of the size or CD3 density on the cell activation using a mathematical simulation, we have recently published [26], and also first estimated the absolute CD3 expression on lymphocytes using the Qifikit® system, in 39 healthy donors. The level of CD3 was $61,050 \pm 25,346$ molecules per cell corresponding to $30,525 \pm 12,623$ TCR/Cell. Thus, we compared the activation kinetics for CD3 levels from 50,000 to 70,000 CD3 mol/cell for one given peptide. For a given size, the simulations have shown that the T cells with higher levels of membrane CD3 reached higher levels of activation (zenith of the peak, Figure 6a). On the other hand, for each CD3 level, the cell size had little effect on the height of the peak (capacity of response) but smaller cells reached more rapid activation (reduced the time-lap) for achieving the zenith of the peak (Figure 6b). Overall, the level of CD3 expression had a predominant influence on activation triggering whatever the size of the cells was.

We then checked that hypothesis experimentally, in an *in vitro* activation model in 13 healthy donors using anti-CD3-CD28 beads that guaranteed equal activation for all T cells. The activation was measured through the induction of Il-2 receptor (CD25) after 30 h, on few V β clonotypes with extreme CD3 levels. The CD4+ T cells (Figure 7 left column) were significantly more activated than CD8+ T cells (Figure 7 right column) that expressed lower levels of CD3. Furthermore, the activation was more efficient on clonotypes that were associated with high levels of CD3 high (V β 8 and V β 9) compared to CD3 low clonotypes (V β 2 and V β 3; $P=0.040$ to $P=0.0003$). The difference was not significant on CD8 V β 1/V β 20 and CD4 V β 2/V β 8 and even reversed in one case (CD4+: the V β 1 cells were more reactive than V β 20 cells: $P=0.007$).

III. Discussion

T cells are usually considered as a homogenous group of cells with similar properties and generally characterized by the mean or median of the whole population neglecting the population variability. The T cells have been separated in two main subsets CD4 / CD8 and more minor subsets such as $\gamma\delta$ T, Th1/Th2, regulatory or Th17 T cells but this was only considered on the quality of their functions. The dynamics of the T cell activation is generally considered as similar for all T cells of any subtypes. In this study, we have focused on the large diversity of T cells within any individual sample in terms of membrane expression of their receptors. We observed that variability was constant among healthy donors as well as among patients with immune disorders. It cannot be attributed to experimental error or variation as

instruments have very narrow signal CV (<2%) when standardized quality assurance beads were tested [37].

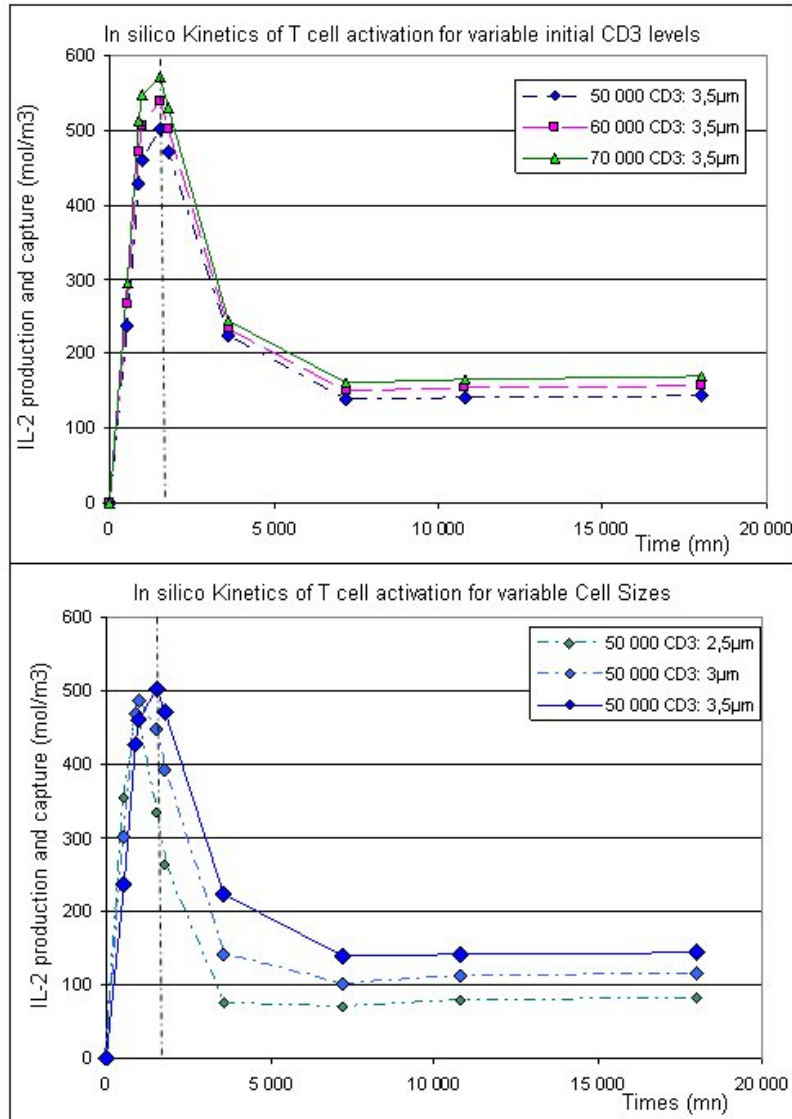


Figure 6: In silico simulation of the T cell activation kinetic for different initial CD3/TCR densities (25-35 000 CD3-TCR complexes per cell) and cell sizes (radius 2.5 to 3.5µm). Activation induced IL-2 and its receptor production. IL-2 binds to its receptor. The complex internalization is considered as the decision parameter for entry in cell cycle. The peak height is the major parameter reflecting the intensity / optimal time for T cell activation as it could be checked using data from ago-antagonist peptides. CD3 level was related to the intensity of the signal while cell size was related to the time delay.

It cannot be due to technical factors as the cells were prepared in the same way, in the same tube, with the same reagents and the individual cells were compared within the same sample, with the same labeling. Furthermore, the differences were very reproducible between different samples.

The T cell diversity we have observed was large enough to have functional significance. Indeed, it has been largely demonstrated [3-5, 10, 15, 17, 20] that the specific T cell activation is directly dependent on the amount of antigens, the amount of TCRs and their mutual affinity. As soon as the T cell has recognized its specific peptide, the TCR-CD3 involved in the binding are activated and internalized.

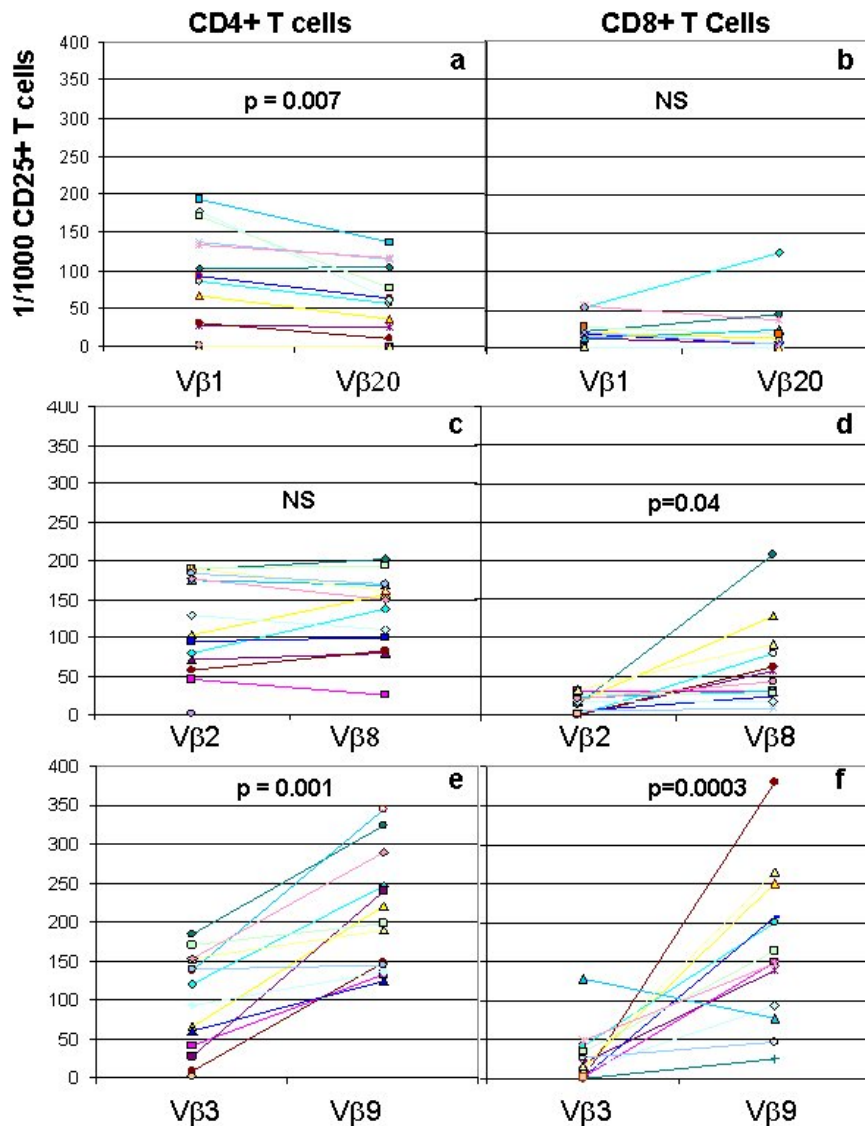


Figure 7: Fresh peripheral blood mononuclear cell from 13 blood donor were activated with CD3-CD28 beads for 30 h. The activation level was measured using CD25 PE-Cy5 on CD3 APC-Cy7, CD4 PE-Cy7 and CD8 APC T cells for the different clonotypes: Vβ1PE/Vβ20FITC; Vβ2FITC/Vβ8PE and Vβ3FITC/Vβ9PE. These clonotypes were chosen for their extreme CD3 levels of expression: Vβ1 (7,366+1,236 / 5,857+841); Vβ2 (8,023 +1,140 / 7,368 + 886); Vβ3 (7,980 +1,628 / 6,947 +1,315); Vβ8 (12,845 +1,695 / 10,457 +1,732); Vβ9 (12,435 +2,093 / 10,430 +1,568); and Vβ20 (11,768 +1,510 / 9,870 +1,174) on CD4/CD8 respectively.

A minimal number of TCR need to be activated to reach the level of activation that is sufficient to make the decision for the cell function and fate (either proliferation, or anergy or activation induced apoptosis).

The number of p-MHC complexes presented by the dendritic cells is less critical because the TCR binding is very brief and several TCR can sequentially bind one single p-MHC complex (serial engagement). However, if the number of CD3-TCR inside the synapse is not sufficient, more are recruited from the outside the synapse. The surface transfer of the receptor takes time and the distance for the transfer rises with the cell size. Thus, the initial number of CD3 certainly influences the speed of T cell specific activation. We tested this hypothesis using computer model we have developed recently [26]. This model was not completely achieved because many parameters that are involved in the activation process still need to be precisely determined. However, preliminary comparisons of the calculated data with experimental data

obtained from the literature [6, 19, 22, 23] have shown that this model was realistic. In the present study, we have used this computer simulation choosing a set of initial parameters (agonistic peptide, cell sizes and CD3 levels of expression) that are close to the conditions *in vivo*. These simulations show that the initial number of CD3 available significantly influences activation in the same order of magnitude as the differences observed with agonistic and antagonistic peptides. We confirmed the functional significance of the initial level of CD3 expression experimentally. T cells were challenged with an APC. Beads coated with anti-CD3 and anti-CD28 should bind equally all T cells, regardless of the TCR structure or avidity for the p-MHC complex. Analysis the early activation effects (membrane CD3, CD25, CD69 expression), during the first 30 h before first cell division because the levels of expression of receptors and the cell size cannot be simply compared between activated mother and new daughters. We compared different clonotypes within the same samples so the cell preparations were performed in the same conditions, in the same well in order to reduce any technical biases. Experimental artifacts were minimized because T cells were exposed randomly, and with equal probability, in the same environmental conditions (same well, same environment) to the most physiological *in vitro* polyclonal activation system (anti-CD3/anti-CD28 on beads). Activation of each clonotype was compared in duplicate and with their unactivated homologs. The labeling should not interfere with the results and high / low CD3 groups were tested with alternative FITC/PE combinations.

The CD3 expression is related to the cell size but the relationship was not linear and cells with high FSC had much more CD3. This was confirm on comparison of FSC values between sections of increasing CD3 levels and confirmed by measuring the individual CD3/FSC ratio. The physical study of FSC reveals that FSC is directly related to the square diameter of the particles, that is, the cell surface. This allowed us to relate the CD3 expression to a surface density. The real cell surface can be calculated but we had many difficulties to count the absolute number of CD3 on different cell subsets. The Qifikit® is the best way to measure it but it uses an indirect labeling method that is not compatible with multiple color analysis. This could have been solved with sorting of different T cell subsets but it would have needed a CD3 labeling that make quantization impossible.

There are physical arguments to explain a relationship between the level of CD3 available and T cell reactivity. A sufficient recruitment of CD3/TCR complex is needed in the synapse in order to reach a sufficient level of signal transduction [7, 12, 17-19, 40]. The number of p-MHC has low limiting role because of possible serial engagement of different TCR on the same p-MHC complex [17-20]. The number of receptors in the synapse is not sufficient to reach that threshold and more molecules are needed from the rest of the membrane. The recruitment is due to membrane transfer of the molecule, at linear speed [13]. It is reasonable to consider this speed as constant for different cell types because it depends on membrane constituents and cytoskeleton motion that is considered common for all T cells. On larger cells, the molecule pool is larger (higher fluorescence intensity) but the transfer takes more time to reach the synapse. In the model, we have used medium cell sizes (radius from 2.5 to 3.5 μm) and CD3 initial densities (50 to 70,000 CD3 ϵ molecules corresponding to 25-35,000 CD3/TCR complexes per cell) and a constant surface speed observed from published experiments [13]. Overall, our theoretical and experimental data have proved that CD3 expression variability has functional implications. Opposite conclusions were raised regarding **GPIIb-IIIa** molecular density that was not correlated to aggregability of L-platelets [41] *In vitro* activation of T cells was repeatedly more efficient on clonotypes with High CD3 levels compared with clonotypes with low CD3 levels. This was not unequivocal as all clonotypes were not tested and some clonotypes did not show significant differences.

Is CD3 level variability related to cell fate? Intriguing but repeatedly confirmed, that CD3 expression was significantly different on T cell subsets. Accordingly, CD4+ T cells (with higher CD3 expression) are more frequently activated in stimulation tests. The relationship with clonotypes was more surprising. The TCR design is a stochastic process that should not

interfere with the cell physiognomy. This discrepancy was already known for $\gamma\delta$ T cells but the ontogeny, the recognition skills and physiological role of these cells are very different from conventional $\alpha\beta$ T cells. How could CD3 expression and activation potential be related to the clonotype? Because the V β segment is involved in binding p-MHC complexes, it is possible that the T cells adapt their CD3 level and size with their avidity for the MHC and / or ligand. T cells could use physical means for a better adaptation of its reactivity to the frequency or intensity of the challenges. On the other hand, considering that the clonotype determination is relatively hazardous for one given peptide/MHC complex, our results suggests some individual variability in the responsiveness to a given antigen according to which TCR clonotype is chosen. The clonotype related mechanism could be influenced by the MHC haplotype, the peptide itself and TCR avidity for the p-MHC complex. The other co-stimulation molecules (CD4 or CD8, CD28, ...) should not strongly interfere because they are common to all cells. Regarding to cytometry data interpretation, membrane density of receptor on cell surface deserves attention because of possible functional significance but one must consider that an increase of mean fluorescence intensity of a cell population does not necessarily mean that surface density is increased on all individual cells [42].

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