FoxP3+ Tregs cells sensitivity to low doses of IL-2 can be rapidly and easily monitored by p-STAT-5 detection in ex-vivo whole blood samples.

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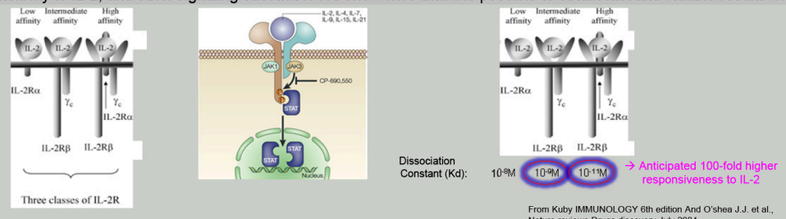
Introduction

Background: Interleukin-2 (IL-2) is a key player in the immune system, with a dual effect: A high dose of IL-2 is known to be a broad activator of most lymphocytes, whereas a low dose of IL-2 is involved in regulatory functions. This effect is associated to the existence of several receptors that can bind IL-2 including the "high affinity receptor constituted of the CD25 (IL2-Rα chain), together with the common IL2-Rγ chain and IL2-Rβ chain and the "intermediate affinity receptor" constituted of the common IL2-Rγ chain and IL2-Rβ chain. It has been shown that the "high affinity receptor" has 10 to 100 fold more affinity for IL-2 than the "intermediate affinity receptor". Therefore CD4+ regulatory T cells (Tregs) expressing CD25 are supposed to respond to lower doses of IL-2 in comparison to CD25neg lymphocytes. The response to IL-2 by T cells could be rapidly detected by looking at the signaling cascade induced by the binding. Unfortunately Phospho-epitopes detection by flow cytometry is tedious. Currently it requires dedicated buffers and protocols, a total work time of 2 to 4 hours, 37°C and ice incubations, and 4 to 8 washing steps. The reference method requires methanol, which is toxic to the user and harmful to certain epitopes, compromising some multi-parametric analysis. For example, the FOXP3 marker is incompatible with "harsh" phospho-epitope detection methods, rendering even more challenging the study of regulatory T cell (Treg) functions. Furthermore, current methods are not optimized for the direct staining of whole blood samples adding some steps for cell preparation. In this respect, there is a strong need for a preparative method allowing the easy monitoring of the Tregs sensitivity to IL-2, and other signaling cascades. We show here that It is possible to obtain fast and reliable results within less than 2 hours.



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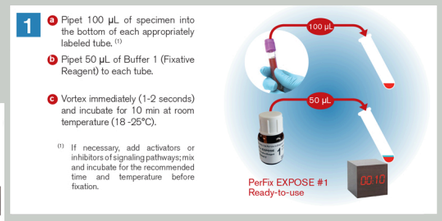
Materials and methods

Materials: We used a new reagent kit; PerFix EXPOSE* (Phospho Epitopes Exposure Kit), which uses multiple innovations in the permeabilization, staining, and wash steps required for intracellular staining. It is devoid of methanol and supports staining of all common phospho-epitopes with one single procedure of about 1 hour. Since many extra-cellular markers can be combined together with the anti-phospho markers, we evaluated also various FoxP3 clones and conjugates, and tried some procedure optimizations. The best conditions were then used to analyse the ex-vivo functionality of FoxP3+ cells, including IL-2 pathway signalling. Samples were acquired on Gallios* cytometer (3 lasers : 10 colors) and data were analyzed with Kaluza* software (Beckman Coulter).

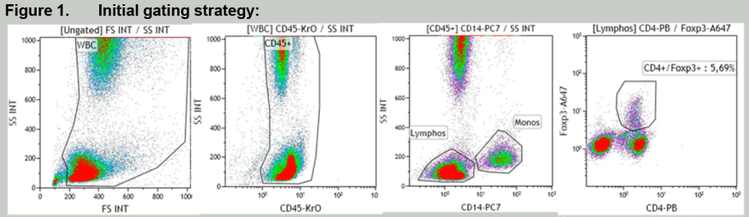
Methods: PerFix-EXPOSE procedure:



12 min. hands-on time,
47 to 70 min. total time.

Results:

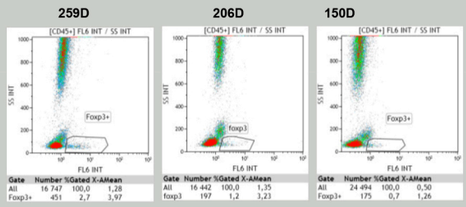
Figure 1. Initial gating strategy:



Gate	Number	%Gated	Y-AMean
All	30 974	100,00	1,84
CD4+/Foxp3+	1 763	5,69	8,56

1. **The scattergram** obtained with this method shows a very good separation of neutrophils from monocytes, monocytes are often very close to lymphocytes.
1. **CD45** staining confirms that debris are almost all excluded in the scatergram, rendering CD45 not essential for purity.
2. **CD14** is useful to easily isolate monocytes out of the lymphocyte cluster.
3. **CD4** is wiped out by the fixation/permeabilization procedure (not shown) and must be used prior fixation. Here is an example showing that a brief incubation (2-5min.) is sufficient to visualize CD4+ cells, even with a dim dye such as Pacific Blue (PB). Other brigther dyes such as FITC, PE or PC7 have been similarly used and worked very well.
4. **FoxP3** staining is then evaluated by recording the % of positive cells among lymphocytes and the Signal-to-Noise ratio (S/N) observed between CD4+FoxP3+ cells and CD4+FoxP3- cells.

Figure 2. FoxP3 clones evaluation:



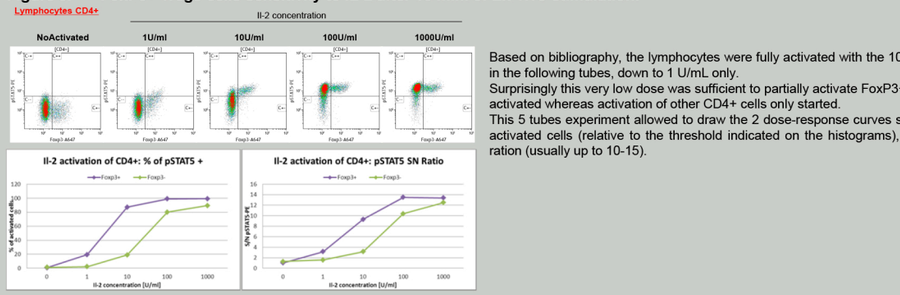
Gate	Number	%Gated	X-AMean
259D All	16 747	100,0	1,28
259D Foxp3+	451	2,7	3,97
206D All	16 442	100,0	1,35
206D foxp3-	197	1,2	3,23
150D All	24 494	100,0	0,50
150D Foxp3+	175	0,7	1,26

3 commercial clones were evaluated (all conjugated to A647), with the PerFix EXPOSE procedure. All were titrated (2x, 1x, and 0,5x), 2 incubation time (30min and 60 min). Only the 259D clone produced satisfactory results, the best representative result of each clone is shown.
Data not shown: No improvement was brought by other common methods such as an extra PBS wash (as recommended in the PerFix-**nc*** procedure for FoxP3), or addition of blocking serum (mouse or human, as recommended by other reference procedures).
As a summary, the optimized conditions for use of FoxP3 in the PerFix EXPOSE procedure are: Clone 259D – Alexa647 from Biolegend, 5µL, incubate 30 min.

N=5 tubes	Mean	SD	CV%
S/N Foxp3 (CD4+)	5,8	0,27	5%
Number of Foxp3+ (CD4+)	1637,6	83,63	5%
% Of Foxp3+ (CD4+)	15,5	0,13	1%
% Of Foxp3+ (total lympho)	5,5	0,19	3%

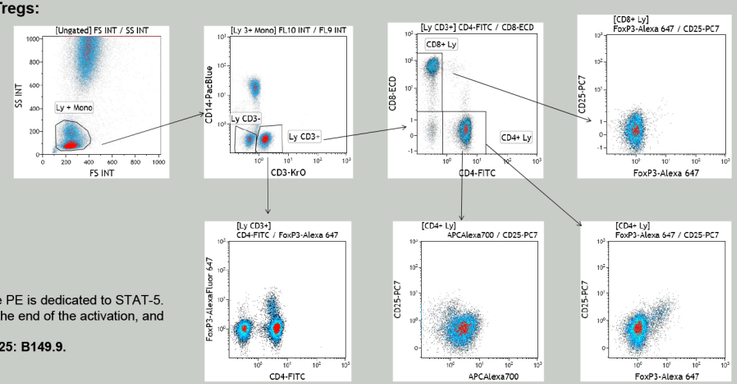
We estimated the variability of the assay by staining the same sample in 5 tubes: The calculated CV% are very good and similar to any surface staining experiment.

Figure 3. FoxP3+ Tregs cells sensitivity to IL-2 after 15 min. of Ex-vivo stimulation:



Based on bibliography, the lymphocytes were fully activated with the 1000U/mL dose, and we titrated down IL-2 in the following tubes, down to 1 U/mL only.
Surprisingly this very low dose was sufficient to partially activate FoxP3+ cells. At 10 U/mL all FoxP3+ cells were activated whereas activation of other CD4+ cells only started.
This 5 tubes experiment allowed to draw the 2 dose-response curves shown below: either considering the % of activated cells (relative to the threshold indicated on the histograms), or considering the STAT-5 signal/noise ration (usually up to 10-15).

Figure 4. Addition of other markers commonly used with Tregs:

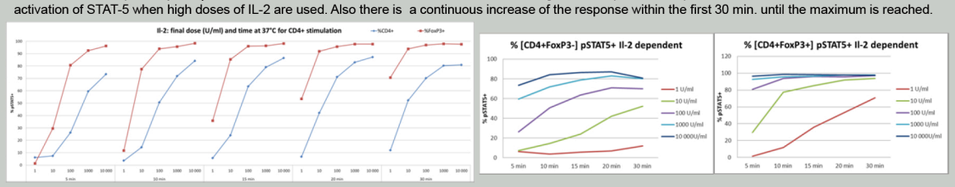


We evaluated the possibility to add CD3, CD8, CD25.
CD45 was removed since it is not necessary for the gating purity
CD14 was shifted from PC7 to Pacific Blue with good result
CD3 staining was weak with the Krome Orange dye, but still visible
CD8 ECD works perfectly, as with any other dye.
All these markers were added AFTER the fix/perm steps.

CD25 must be used with bright dyes such as PE or PC7, here we chose PC7 since PE is dedicated to STAT-5.
As for CD4, CD25 must be used PRIOR to fixation, it was added 5 minutes before the end of the activation, and it seems not to interfere with the STAT-5 activation (Data not shown).
Recommended Clones: CD3: UCHT1, CD4: 13B8, CD8: 8H8, CD14: RMO52, CD25: B149.9.

Figure 5. Dose and time-response curves

In order to verify the kinetics of STAT-5 activation by IL-2, the titration of IL-2 was reproduced for various incubation time (5, 10, 15, 20, 30min.), on the same donor.
Results confirmed perfectly the previous ones at 15 minutes, and extended them showing a time-dependant response: 5 min. after the addition of IL-2 there is already a very strong activation of STAT-5 when high doses of IL-2 are used. Also there is a continuous increase of the response within the first 30 min. until the maximum is reached.



Summary:

PerFix EXPOSE* allows the simultaneous detection of some antigens that are otherwise incompatible, such as FOXP3 and phospho-STAT epitopes. Using this new kit, an IL-2 dose-response curve could be easily generated directly from fresh whole blood within less than 2 hours (including activation time). This confirmed on normal donors a 10- to 100-fold difference in sensitivity to IL-2 between Tregs and other T cells.