Chapter 4

Cryopreservation does not alter the frequency of regulatory T cells in peripheral blood mononuclear cells.

D. Van Hemelen
J.N.G. Oude Elberink
J. Heimweg
A.J.M. van Oosterhout
M.C. Nawijn

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Sir,

With great interest we read the recent article in the Journal of Immunological Methods by Elkord *(2009)* in which the author reports a reduction of human regulatory T (Treg) cell numbers in peripheral blood mononuclear cells (PBMCs) after cryopreservation [1]. Treg cells were identified by flow cytometric measurement of $CD25^{hi}$ or Forkhead Box P3 (FoxP3) expression in $CD4^+$ T cells. CD25 and the transcription factor FoxP3 are known to be expressed by functionally suppressive Treg cells [2], but are not specific for this subset of $CD4^+$ T cells, as recently activated T cells are also able to transiently express CD25 and FoxP3 [3]. We are interested in the application of cryopreservation of PBMCs, as it allows the simultaneous analysis of samples obtained at multiple time points during a clinical trial. Given the pivotal role of Treg cells in the treatment of allergic diseases, we have in the recent past extensively studied the effects of cryopreservation on T cell subsets, and in particular on $CD4^+$ T cells expressing CD25 and FoxP3, allowing direct comparison to the data published recently by Elkord *(2009)*[1].

In contrast to the results reported by Elkord *(2009),* we do not detect substantial differences in the expression of FoxP3, or the cell-surface marker CD25 within the $CD4^+$ T cell population between freshly isolated and reconstituted cryopreserved PBMCs [1]. Here, we show data derived from PBMCs obtained from three independent individuals after receiving informed consent, analyzed before, and after minimum one week of cryopreservation (figure 1). Elkord *(2009),* as well as Costantini *et al.,* reported a decrease in the percentage of $CD4^+$ T cells after cryopreservation [1,4]. Our data suggests a similar reduction in $CD4^+$ T cell numbers after cryopreservation (48.2 ± 3.8% to 41.8 ± 6.3 %, figure 1b), but the reduction in $CD4^+$ T cell numbers is not statistically significant in this small sample group. In contrast, the percentages of $CD4^+$ T cells expressing CD25, or FoxP3 were not altered after cryopreservation (figure 1). Looking at the recovery, the fraction of $CD25^{hi}$ (85%) and $FoxP3^+$ (82%) $CD4^+$ T cells recovered after cryopreservation is comparable to the recovered fraction of total $CD4^+$ T cells (86%). These data underscore the absence of a specific loss of $FoxP3^+$ or CD25hi subpopulations within the $CD4^+$ T cell fraction. Additionally we analyzed the expression of the cell-surface markers CTLA-4 and GITR within the $CD4^+$ T cell population, and also here we could not detect any differences between freshly isolated PBMC and those recovered after cryopreservation (data not shown).

The observed decrease in the percentage of $CD4^+$ $FoxP3^+$ T cell population in the data published by Elkord (2009, and Figure 1 and 2 therein), seems clear, and is observed in every single individual tested. The most likely explanation for these differences is a slightly different approach to cryopreservation. In contrast to the 'freezing medium' used by Elkord *(2009),* consisting of 90% fetal bovine serum (FBS) and 10% dimethyl

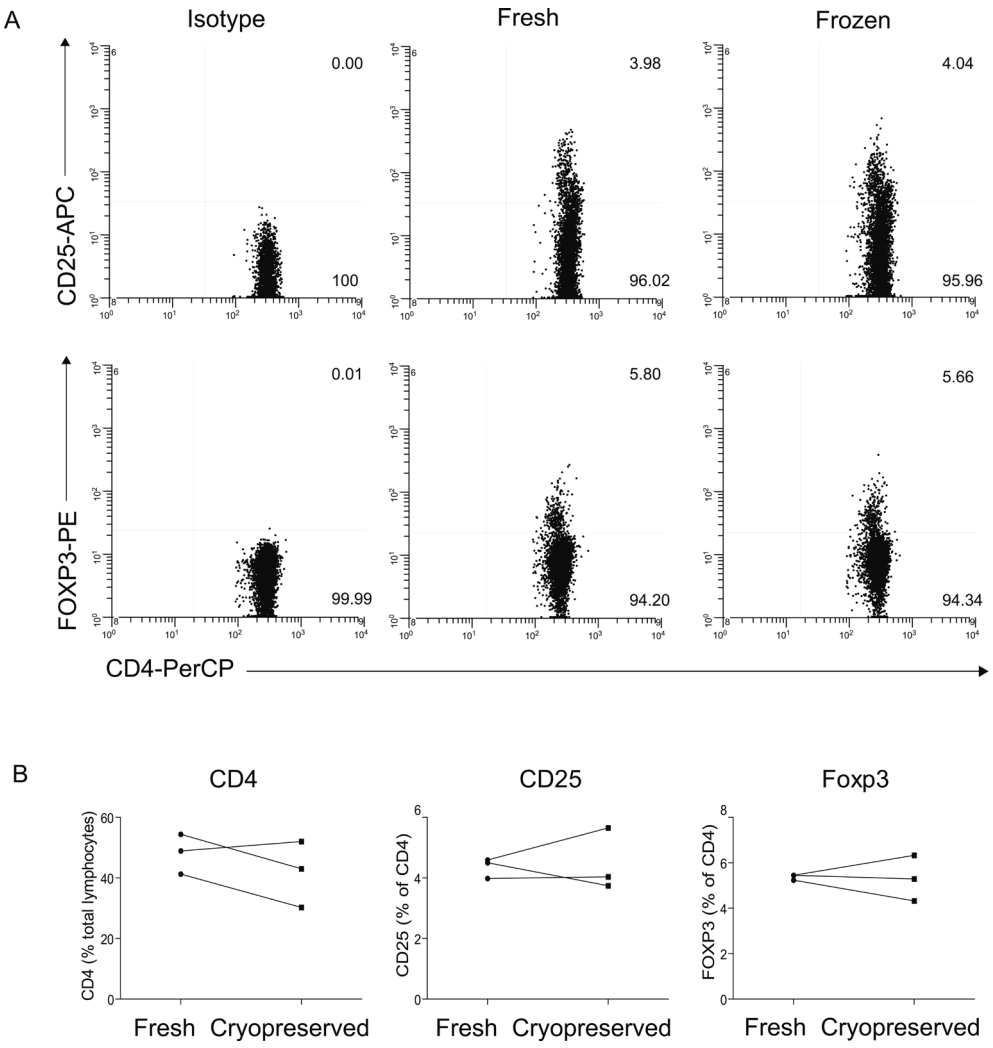

Figure 1. Expression of T cell markers CD4, CD25, and FoxP3 as determined by 3 color flow cytometry in freshly isolated and reconstituted cryopreserved PBMCs. Representative dot plots from freshly isolated and reconstituted cryopreserved PBMCs are shown for CD4 gated T-cells, stained for isotype control, CD25, and FoxP3 (A). The percentages of CD4+ cells in the total lymphocyte population, and CD25+ and FoxP3+ cells in the CD4+ population are shown before and after cryopreservation from three independent individuals (B).

sulfoxide (DMSO), our 'freezing medium' contains 50% FCS, 40% HBSS (Lonza/Bio-Whittaker) and 10% DMSO (Sigma-Aldrich, St Louis, MO, USA). After isolation, the PBMCs are first resuspended in FCS/HBSS medium alone, whereafter the DMSO is gently added to the suspension, instead of immediately dissolving the PBMCs with the freezing medium already containing DMSO. The freezing cycle used in both protocols seems identical, since we freeze our samples in Cryo Preservation Modules (Stratagene, La Jolla, Canada), as do the authors, rendering a controlled cooling rate of - 0.4-0.6 °C/minute when placed at -80 °C. After 24h, the frozen vials were transferred into liquid nitrogen.

To reconstitute the frozen samples, Elkord *(2009)* transferred the rapidly thawed samples drop-by-drop to 10ml of 10% FBS in RPMI [1]. In contrast, we gradually dilute the contents of the rapidly thawed cryovials with 10 ml of 37 °C culture media (RPMI-1640 (Lonza/Bio-Whittaker) supplemented with 100 U/ml penicillin, 0.1 mg/ml Streptomycin, and 50 μM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA)). This gradual dilution is performed by first adding 1 ml, followed by a drop wise addition of the remaining 9 ml of medium, which is important to avoid osmotic stress, reducing cell damage [5]. After this first washing step, we wash our cells a second time by gradually adding 20 ml of culture medium, while the centrifugation speed between the washing steps was limited to 380g.

For analysis, the cells were resuspended in PBS (10^6/ml), and analyzed by an antibody staining procedure identical to the paper of Elkord *(2009)* [1]. As both studies used an identical method to detect human regulatory T cells (Human regulatory T cell staining kit, eBioscience, San Diego, CA, USA) in combination with similar CD25-APC / FoxP3-PE (clone PCH101) antibodies, the differences in Treg cell recovery after cryopreservation between both studies are most likely based on the processing of the PBMCs during or after cryopreservation. Although we did not directly compare our cryopreservation protocol to the one from the Elkord *(2009),* paper, we tend to conclude that the sensitivity of Tregs to cryopreservation will depend on the way these cells are treated during the process instead of on the process of cryopreservation *per se*.

Another point of discussion is the gating strategy used by Elkord *(2009)* to study CD25-expressing $CD4^+$ T cells. In Figure 1A, the authors depict dot plot diagrams showing a CD4 (X-axis) versus CD25 (Y-axis) staining [1]. The numbers shown in these plots are the percentages of $CD4^+CD25^{hi}$ T cells within the whole lymphocyte population. These numbers represent the recovery rather than the fraction of $CD4^+$ T cells expressing CD25. Since the authors also reported a decrease in $CD4^+$ T cell numbers after cryopreservation, it can readily be anticipated that a similar reduction in the percentage of $CD4^+CD25^+$ expressing T cells within the whole lymphocyte population will be observed. In our perspective, the $CD25^+$ T cells should have been analyzed as a

fraction of the total number of $CD4^+$ T cells to avoid this side effect of analysis, as has been done by the authors in the analysis of the FoxP3 expressing T cells (Figure 1C) [1]. Last but not least, it is important to mention that we do not want to discharge the key message within the paper of Elkord *(2009),* which is to perform consistent and comparative analyses on cryopreserved and freshly isolated PBMC samples [1]. Although cryopreservation *per se* does not significantly change the number of $CD25^{hi}$ or $FoxP3^+$ T cells within the $CD4^+$ population, it can not *a priori* be assumed that cryopreserved and freshly isolated PBMC samples are identical, neither in composition nor in cell function. We certainly do not attempt to discard this notion with our letter, but instead we show that it is possible using the protocol specified above, to retain a Treg cell population after cryopreservation that is identical in number to freshly isolated PBMC. It remains to be established whether cryopreservation affects Treg cell function.

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