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Challenges in cryopreservation of regulatory T cells (Tregs) for clinical therapeutic applications

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ABSTRACT

Promising results of initial studies applying *ex-vivo* expanded regulatory T cell (Treg) as a clinical intervention have increased interest in this type of the cellular therapy and several new clinical trials involving Tregs are currently on the way. Methods of isolation and expansion of Tregs have been studied and optimized to the extent that such therapy is feasible, and allows obtaining sufficient numbers of Tregs in the laboratory following Good Manufacturing Practice (GMP) guidelines. Nevertheless, Treg therapy could even more rapidly evolve if Tregs could be efficiently cryopreserved and stored for future infusion or expansions rather than utilization of only freshly isolated and expanded cells as it is preferred now. Currently, our knowledge regarding the impact of cryopreservation on Treg recovery, viability, and functionality is still limited. Based on experience with cryopreserved peripheral blood mononuclear cells (PBMCs), cryopreservation may have a detrimental effect on Tregs, can decrease Treg viability, cause abnormal cytokine secretion, and compromise expression of surface markers essential for proper Treg function and processing. Therefore, optimal strategies and conditions for Treg cryopreservation in conjunction with cell culture, expansion, and processing for clinical application still need to be investigated and defined.

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1. Introduction

Regulatory T cells (Tregs) have been described as key regulators in various immunological processes, particularly in the suppression of immune response, as well as in the induction and maintenance of self-tolerance. Since their discovery, there has been great interest in using Tregs as immunotherapy. Many animal studies have proven that such therapy is feasible and effective in autoimmunity [1–4], allogeneic transplantation [5,6] and Graft Versus Host Disease (GVHD) [7–10]. In the last 3 years, results of the first clinical trials with Tregs have been published. Three of those publications reported that Treg therapy was safe and might be an effective tool to prevent or treat GVHD [11–13]. Another recently published study demonstrated that autologous Treg infusion can be safely used to preserve β -Cell function and may delay onset of Type I diabetes in children [14]. These findings have increased interest in utilizing Tregs as therapy in solid organ transplantation as well as for the treatment of autoimmune disorders. Treg cellular therapy in humans is especially challenging, since the number of these cells

in peripheral blood is low, and substantial numbers of Tregs are needed for the prevention of graft rejection or halting autoimmune reactions. The exact critical Treg number is unknown, but based on animal models it is believed that the ratio of Tregs to conventional T cells should be between 1:1 and 1:2, which would require billions of Tregs per one infusion to effectively suppress the immune system [15]. However, recent technical improvements in Treg isolation and *ex-vivo* expansion protocols [16–22] have been developed towards overcoming the relative paucity of Tregs and have made it feasible to obtain sufficient numbers of fresh cells for clinical adoptive transfer therapy. The main source of the Treg has been a patient's own blood (drawn directly from a blood vessel or as a product of leukopheresis) or third-party umbilical cord blood.

Nevertheless, from a practical perspective, therapy with regulatory T cells would be greatly improved if isolated/expanded Tregs or PBMCs/pre-enriched cells for Treg isolation could be stored long-term until they are needed. This approach would allow for more flexibility in terms of planning the therapy, timing of the infusions, and also allow for subsequent Treg infusions, regardless of pharmacological treatment applied after the first infusion [23,24]. However, current knowledge of how the process of cryopreservation may affect Tregs is still limited. Thus, the focus of this review is on findings concerning PBMC/Treg cryopreservation and the possible effects of freezing/thawing on those cells, their biology, and function with relevance to their clinical application.

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2. Treg recovery from the cryopreserved PBMCs

Cryopreservation of PBMCs is performed routinely for subsequent immunological, laboratory analysis, when it is not possible to use the cells shortly after blood sample collection. From a Treg therapy perspective, PBMCs can be frozen and banked for subsequent Treg isolation, processing and infusion to the patient. Assessment of Treg frequency in PBMCs is usually done by flow cytometry after surface (CD4⁺, CD25^{high}, CD127^{low}) and intracellular (FoxP3) staining. There have been several studies, which evaluated the effect of cryopreservation on Treg detection in PBMCs based on the above markers. A study in 2008, showed that cryopreservation decreases the proportion of CD4⁺CD25⁺ Tregs in PBMCs in samples from either healthy or HIV-1-infected individuals [25]. The authors suggested that a change in the proportion of CD4⁺CD25⁺ cells between fresh and thawed PBMC samples is due to significant changes in surface expression of CD25⁺ on processed CD4⁺ T cells, probably caused by increased apoptotic susceptibility of activated T cells which express CD25 [25]. Elkord also noticed a significant reduction in Treg recovery from cryopreserved PBMCs comparing to freshly isolated cells; of note, he assessed it also looking at intracellular staining of FoxP3 in addition to the surface markers CD4 and CD25. In samples from 6 healthy volunteers, the mean % of CD4⁺FoxP3⁺ cells in fresh samples was 5.58% (CI = 3.39–7.77) and decreased to 3.57% (CI = 1.94–5.22) ($p = 0.0016$) following thawing after 3 weeks of cryopreservation. The author hypothesized that these results were due to Treg fragility under cryopreservation conditions [26].

On the contrary, Van Hemelen et al., after analysis of frozen/thawed PBMCs from 3 healthy donors, did not detect a significant decrease in expression of CD25 and FoxP3 within the CD4⁺ population after cryopreservation. Also, expression of other Treg surface markers, CTLA-4 and GITR, in the CD4⁺ population, was not different between freshly isolated and cryopreserved PBMCs. Van Hemelen et al. suggested that results observed by Elkord [26] were a result of the specific cryopreservation media method used in that study which consisted of 90% fetal bovine serum (FBS) with 10% Dimethyl sulfoxide (DMSO). In contrast, Van Hemelen's group used medium containing of only 50% fetal calf serum (FCS), but with 40% Hank's Buffered Salt Solution (HBSS) and 10% DMSO. Moreover, DMSO was added gently after re-suspending cells in HBSS/FCS, instead of immediately re-suspending the PBMCs in the medium already containing DMSO. In addition, there were differences in the thawing methods between those two studies: Elkord added thawed cell suspension drop-by-drop to 10 ml of RPMI medium with 10% FBS, while Van Hemelen's group diluted thawed cells with 10 ml RPMI medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 μ M β -mercaptoethanol, adding firstly 1 ml of this medium, followed by drop-wise addition of remaining medium to avoid osmotic stress [27].

Another recent study based on a larger number of samples ($N = 32$ from healthy volunteers and $N = 15$ from HIV-1 infected patients) brought more evidence that cryopreservation compromises Treg recovery regardless of cell processing method. In both, surface and intracellular staining methods, Treg numbers in cryopreserved PBMCs were reduced in comparison to numbers in fresh PBMCs. The frequency of CD4⁺CD25⁺FoxP3⁺ cells from healthy donors was 3.13% in fresh samples, versus 1.26% in frozen/thawed ones ($p < 0.0001$); in HIV-1 infected patients Tregs were 2.68% in fresh samples, versus 0.94% in frozen/thawed ones ($p < 0.0001$). Assessing Tregs by surface markers, the proportion of CD4⁺CD25⁺CD127^{lo/-} decreased from 6.5 to 4.2% ($p < 0.0001$) in healthy subjects and from 7.1 to 3.8% ($p < 0.005$) in HIV-1 patients, despite using a similar freezing/thawing protocol as Van Hemelen's group. The study concluded that the reduction in Treg frequency after cryopreservation was due to selective Treg cell death caused by their susceptibility to apoptosis as they are activated cells expressing CD25⁺. Alternatively, cryopreservation may just impair staining of Treg cell receptor markers and their detection [28].

3. Cryopreservation of isolated/expanded Tregs

Direct cryopreservation of isolated Tregs and simple banking of Tregs could be a very practical solution from a clinical perspective. Current experience with direct Treg cryopreservation is still very limited. Published studies differ in freezing/thawing technique: in some studies freshly isolated Tregs were cryopreserved, in others, Tregs were frozen after *ex-vivo* expansion; many different cell markers and tests were used for cell identification and assessment. Therefore, it is not easy to compare those studies and draw conclusions.

As suggested by Peters et al., it is convenient to perform a leukopheresis procedure weeks/months before the transplantation or treatment and store isolated Tregs until they are needed [24]. Such an approach was studied and the results showed that it is feasible [24]. Tregs isolated *via* CliniMACS (CD8 depletion followed by CD25 enrichment) were cryopreserved in medium containing 20% human pooled serum and 15% DMSO and stored in liquid nitrogen for one year. After thawing, Tregs showed 70–80% viability. Although the suppressive capacity of cryopreserved Tregs was impaired and could not be regained by cell resting for up to seven days in culture medium, it was eventually restored after Treg stimulation and subsequent expansion [24].

Another option is to freeze already expanded Tregs. This can be especially practical, if we have a surplus of cells remaining after the first infusion, which could be used for later therapy. In clinical trials with Tregs isolated from Umbilical Cord Blood (UCB) for the treatment of GVHD ($N = 14$), cells remaining after the first infusion were frozen with Plasma-Lyte ATM (Baxter, Deerfield, IL), 10% DMSO, and human serum albumin, and stored in Cryocyte bags for a planned second infusion on day + 15. Before the second infusion, cells were thawed, diluted with 5% albumin/10% Dextran 40, and checked for post-thaw viability (>50%). After the first infusion of fresh Tregs, a significant increase in frequency and absolute number of Tregs in the peripheral blood was observed. In contrast, after a second infusion of the same number of Tregs which had been cryopreserved instead of fresh ones, such an increase was not observed. Although the mechanism for this is unknown, possible reasons for inability to detect increased numbers of Tregs after the second infusion include poor viability of cryopreserved Tregs *in vivo* after infusion, despite the fact that immediate post-thaw viability exceeded 50%. This hypothesis might be supported by the observation that after overnight culture, the viability of thawed Tregs is deeply compromised (Karolina Golab, unpublished observations). The authors suggested also that this effect could be caused by changes in the homeostatic cytokine environment or pre-sensitization to antigens expressed on the Treg product after the first infusion [12].

Therapy with UCB-derived or other examples of third-party Tregs would be greatly facilitated by the creation of cell banks of cryopreserved Tregs. Third party Tregs were considered for use in pancreas xenotransplantation for diabetic patients, from whom collection of Tregs may represent a challenge [29]. Tchorsh-Yutsis et al. found that freezing and thawing did not adversely affect the function of expanded third-party Tregs by comparing suppressive activity *in vivo* of cryopreserved and fresh Tregs. Thawed Tregs effectively prolonged embryonic pig pancreatic graft survival and function in C57BL/6 mice for up to 8 weeks post-implantation, similarly to freshly isolated Tregs [29].

A more detailed study showed that human Tregs, which were massively expanded by stimulation with cell-based artificial antigen presenting-presenting cells (aAPCs) and subsequently frozen, maintained suppressive function *in vitro* after thawing [18]. The average recovery was ~60%. Cultured Tregs maintained their phenotype (CD4⁺CD127⁻FoxP3⁺) after cryopreservation [18]. With such successful results, authors concluded that a massive Treg expansion might be achieved by re-stimulation with aAPCs and a master cell bank of cryopreserved Tregs can be established and serve as therapy for multiple diseases [18].

Allo/auto-antigen-specific Tregs are considered to be more effective and safe because they are less likely to confer non-specific general immunosuppression as polyclonal Tregs. Dromey et al. demonstrated that auto-antigen-specific CD4⁺ Treg clones could be generated from blood CD4⁺ cells by stimulation with glutamic acid decarboxylase auto-antibody (GAD65) or proinsulin. Subsequently, they can be expanded, freeze/thawed, and could serve as a cell therapy for diabetes mellitus type-1 (T1DM) [30]. After expansion in the presence of phytohemagglutinin and IL-2 and IL-4, auto-antigen specific Tregs were frozen in liquid N₂ in 10% DMSO/90% human pooled serum. After 7–19 months, they were thawed, expanded for 12 days to check proliferation response and their suppressive capabilities. Thawed auto-antigen GAD65-specific Tregs proliferated in response to PHA or anti-CD3 antibodies and IL-2 + IL-4, but remained unresponsive to stimulation by GAD65. What is more, after freezing/thawing and expansion, GAD65-specific Tregs had decreased suppressive capability when comparing to the state prior to their cryopreservation. The authors suggested that reduced suppressive abilities could be explained by repeated re-stimulation by PHA in long-term culture after cryopreservation [30].

4. Effects of cryopreservation on Treg biology and function

Current literature about if and how cryopreservation affects Treg biology and functionality is still very limited. As it is reviewed above, the protocols for Treg freezing/thawing, the time-points of specific steps of the procedure, as well as the assays used to evaluate Treg post-thaw stability and functionality are not unified and differ between studies. In contrast, there are more studies describing the impact of the cryopreservation on PBMCs and based on that, we can learn and speculate on effects of cryopreservation on Tregs.

4.1. DMSO

The most commonly used protocol for cryopreservation is based on freezing cells in 10–20% DMSO in a freezer for short time and subsequently in liquid nitrogen for a long, unattended storage. DMSO is an amphiphatic molecule, which relatively easily penetrates cell membranes [31]. DMSO serves as cryoprotectant preventing the formation of intracellular ice crystals and disruption of cell membranes and osmotic injury during freezing. Still, DMSO has been found to be toxic to animal and human cells in high concentrations. The mode of action on PBMCs seems to be dose- and time-dependent. Exposure to more >2% DMSO for more than 2 h induces cell death and compromises T cell function [32]. However, it seems that concentrations of up to 10% for only 1 h do not affect the cell viability and the functional responses of CD4⁺ and CD8⁺ cells [32]. Nevertheless, the freezing/thawing procedure should be performed with special caution to avoid prolonged exposure of cells to DMSO. Traditional methods assume that low temperatures decrease cell metabolism and protect cells from the toxic effect of DMSO so cells are kept on ice and placed in ice cold freezing medium during processing. However despite that, Kreher et al. showed that after adding freezing medium to PBMCs at room temperature instead of keeping them cold during cryopreservation, the cell recovery rate after thawing was significantly higher in comparison to methods where PBMCs were kept in medium on ice (84% and 54% respectively; $p \leq 0.001$) [33]. The protocol of freezing/thawing plays an important role in achieving high recovery of viable and functional cells. There are a number of factors that should be considered when formulating a cryopreservation protocol such as: speed of diluting cells during thawing [34], temperature of medium used for freezing/thawing [35], cell concentration for freezing, storage temperature of cryopreserved cells, adding DNase to avoid cell clumping during thawing [36], supplementation of conventional medium for freezing with bio-antioxidant catalase, and the membrane stabilizer trehalose [37]. Choice of appropriate protocol for cryopreservation of Tregs plays a critical role towards achieving high recovery of fully functional Tregs after

cryopreservation and all the known factors that affect PBMCs during freezing/thawing should be taken into consideration.

It is known that freezing affects PBMC proliferation, cytokine secretion, and protein and mRNA expression. There are several mechanisms responsible for such effects including oxidative stress, mechanical injury due to ice crystal formation, altered physical properties of cellular structures, osmotic injury, disturbed ion homeostasis due to Na⁺/K⁺-adenosine triphosphatase (ATPase) inhibition, and apoptosis [36]. One of the studies showed that frequencies and type1/type 2 cytokine signatures of antigen-specific CD4 and CD8 human cells are unaffected after freezing/thawing. To test this response, the levels of interferon (IFN)- γ , IL-2, IL-4 and IL-5 were measured by enzyme-linked immunospot (ELISPOT) in CD4⁺ and CD8⁺ cells upon stimulation with antigen [33]. Similar results were observed in a multicenter study in 2009. The median differences in cell frequencies before and after cryopreservation were 0% for CD4 and 1% for CD8, indicating no significant loss of cells. Also, a functional assay, the lymphocyte proliferation assay (LPA) in response to antigens, showed high concordance in results of stimulation indices before and after cryopreservation [34].

The above results are in contradiction with another report looking at cell population composition and functional assays of PBMCs after cryopreservation. PBMC cell populations during analysis were divided into CD4⁺, CD8⁺, monocytes, B cells, and natural killer (NK) cells. No significant changes were observed for recovered CD4⁺ cells and NK cells in contrast to B-cell and CD8⁺ cell recovery. The study aimed at determining if frozen PBMCs are adequate for performing established immunological assays in type 1 diabetes-related research, as well as to test functionality of frozen/thawed PBMCs. Islet antigens were used in validated assays (CD4⁺ ELISPOT, proliferation assay, cellular immunoblotting, class II tetramer assay). In frozen PBMCs, lower response to stimulation with islet antigens was observed in all the T-cell functional assays [35]. Kvarnström et al. reported results which contradict the previous findings by Kreher et al. [38]. IFN- γ , IL-4, IL-5, IL-9, IL-10, and IL-13 responses were measured by ELISA, ELISPOT and/or real-time reverse transcription-polymerase chain reaction (RT-PCR) in the basal state and after stimulation with self-antigens, allergens, and mitogens in several groups of patients with multiple sclerosis, atopic children, non-atopic children, and pregnant women. Results showed significant differences in cytokine profile of PBMCs before and after cryopreservation, both at the protein and the mRNA level, depending upon patient group, cell preparation for T cell assays, stimulus, and cytokine response. The most consistent results were for IL-4: the expression level was decreased in cryopreserved PBMCs independent of mentioned factors. Expression of other cytokines, both at the protein and mRNA levels was also affected by cryopreservation process, but the effect was not consistent and varied among different cytokines, different stimuli, and different patient groups [38]. This issue is of special importance as cytokine secretion is regarded as one of the mechanisms of Treg suppressive activity [39]. That is why dysregulation of IL-10 production upon cryopreservation, which was shown by Kvarnström et al. [38], can be detrimental for Treg functionality.

In addition, Weinberg et al. reported that there are no changes in frequencies of CD4⁺ and CD8⁺ cell populations in PBMCs due to cryopreservation. Out of CD3, CD28, CD38, CD45RA, CD45RO, CD62L, CD95, HLADR markers, [34] they found a lack of stability only for CD62L and CD45RO during cryopreservation. There was a cell frequency decrease in about 31% for CD4⁺CD45RA⁺CD62L⁺ and in about 17% for CD3⁺CD4⁺CD45RO⁺ cells [34]. A similar reduction of CD62L expression was observed by Costantini et al. [40]. The frequency of CD62L positive T cells declined from 65 \pm 9.4% to 41 \pm 16.3% ($p < 0.0001$) in both CD4⁺ and CD8⁺ T cell populations after cryopreservation, causing a decrease in the frequency of 'naïve' (CD45RA⁺CD62L⁺) and 'central memory' (CD45RO⁺CD62L⁺) T cells. From all markers tested: CD3, CD4, CD8, CD57, CD45RA, CD45RO, CD62L, CD28, CD38, CD95, CXCR4, and CCR5, there was only a decline in the percentage of cells expressing CD62L and CCR5 during the cryopreservation [40]. A similar decrease in

CD62L was obtained also among CD34-positive cells [41,42]. Hattori et al. proposed that it is DMSO which activates matrix metalloproteinase (MMP), causing L-selectin (CD62L) to be shed from the cell surface [42]. Pre-incubation of cells with MMP-inhibitor KB-R8301 and subsequent incubation with DMSO abolished the decrease in the CD62L⁺ population [42]. Moreover, in both studies, the effect is transient and CD62L is re-expressed after overnight cell culture [41,42]. Such a mechanism explaining loss of markers related to their internalization or shedding from the cell surface rather than a selective killing of CD62L positive cells was also proposed by Costantini et al. [40]. Decreased expression on L-selectin (CD62L) and CCR5 after cryopreservation could have a significant detrimental impact on Treg function. Tregs lacking expression of CCR5 were far less effective in preventing lethality from GVHD [43] and a lack of L-selectin compromises their trafficking capabilities, localization, and consequently the compartmentalization of Treg-controlled immune responses [44]. It was shown that CD4⁺CD25⁺CD62L⁺ cells can migrate towards secondary lymphoid tissues and, in contrast to CD4⁺CD25⁺CD62L⁻ cells, can effectively prevent the development of autoimmune diabetes in a mice model [45]. Therefore, decreased expression of CD62L and CCR5 related to cryopreservation could be very important from a clinical therapy perspective.

In summary, cryopreservation can also affect certain functions of cells as has been reported by many studies on PBMCs and can be more detrimental to certain subpopulations of PBMCs [35], have an impact on the profile of produced cytokines [35,38], impair PBMCs response to antigens [35] and alter the expression of various cell surface markers [34,35,40]. All these observations should also be taken into account when the effects of cryopreservation on Tregs are considered. Therefore, effects of cryopreservation on Tregs should be further investigated.

5. Different strategies of Treg processing and cryopreservation for clinical application

From a clinical perspective, it is desirable to retrieve autologous Tregs from patients prior to required pharmacological therapy (which might alter their properties), then expand Tregs if necessary, test and cryopreserve cells, and have them ready when timing for clinical infusion is optimal. This approach would allow also for consequent infusions in the same patient regardless of types of therapies applied in the meantime. In case of third-party Tregs, the rationale for cryopreservation is similar.

Technically, in one approach, we could retrieve PBMCs from the patient, freeze them, store and thaw shortly before the planned infusion, then isolate Tregs and expand them *ex vivo*. As described above, the main disadvantage of this approach is that that Treg frequency in PBMCs after cryopreservation can be substantially decreased as it was shown by Seale et al. [25], Elkord [26] and Sattui et al. [28]. Moreover, cells immediately after stressful thawing are exposed to additional detrimental conditions during the sorting procedure. They are exposed to: antibody/bead complexes, passage through the channels of the sorter or magnetic columns, and multiple washes and centrifugation. Not only the viability but also the function of the cells after such intense processing might be substantially compromised. Moreover, we can also encounter difficulties with Treg isolation due to the instability of Treg markers (receptors) after cryopreservation, which are critical for Treg separation from other cell populations, as was suggested by Sattui et al. [28]. Summing up, number of Tregs achieved after isolation from cryopreserved PBMCs can be insufficient for infusion.

To avoid the above disadvantages, in an alternative approach, Tregs could be freshly isolated or isolated and *ex vivo* expanded first, then cryopreserved, stored and thawed just prior to infusion. The feasibility of this approach was presented in several studies, as Tregs still expressed immunosuppressive properties after such processing [12,18,24,29,30]. In this approach, the recovery rate and variability might be minimally affected. Even though immediately after

thawing viability of the Tregs may be high, the quality of the cells might be still affected. In this case, the biological effect of those Tregs might be compromised if infused immediately after thawing. Many of those cells might have been already in an apoptotic state and the number of non-viable cells could be increasing with time as it was shown in subsequent to thawing *in vitro* cultures. Cell membrane disintegration and cell lysis or adherence to the culture dish were seen [46]. Moreover, *in vivo*, apoptotic cells are recognized by macrophages and rapidly engulfed and digested, in a process referred to as clearance [46]. The apoptosis of Tregs could have been a reason for lower numbers of circulating Tregs detected after infusion of cryopreserved Tregs versus fresh ones in the previously mentioned clinical trial [12].

Therefore, one of the solutions to overcome above problem could be subsequent Treg *ex-vivo* expansion with stimulation after cell thawing, regardless of whether Tregs were already expanded, before cryopreservation. In this case, poor viability after cryopreservation may be overcome by natural selection of only viable cells during culture expansion, allowing one to obtain the desired amount of high quality cells for clinical infusion and eliminating poor quality ones. Our own results confirm the feasibility of such an approach: *ex-vivo* expanded Tregs after cryopreservation, thawing, and subsequent culture expansion sustained their proliferative abilities, maintained their phenotype of CD4⁺CD25^{hi}CD127^{-/lo}FoxP3⁺, and their ability to suppress T effector cells in a proliferation assay (Karolina Golab, unpublished data).

Detrimental effects of cryopreservation on Treg function and viability prompt us to look for improvements in the cryopreservation technique. One of the factors to be considered is freezing rate. It has been shown that controlled-rate freezing improves platelet and lymphocyte recovery rates versus “dump” (uncontrolled-rate) freezing techniques [47]. Therefore currently, application of controlled-rate freezers is preferable and further optimization of freezing rate programming should be studied. From a clinical perspective, the other important aspect is using appropriate containers for storage of cryopreserved cells. If Treg infusion is considered immediately after thawing, cryobags are preferred over cryovials since they allow for closed system processing without exposing thawed cells to the environment. Otherwise, if Treg isolation, culture, or expansion is involved prior to or following cryopreservation, current Good Manufacture Practice rules apply for cell processing in the cGMP facility. Long-term cell storage in liquid nitrogen is preferred over a –70 °C freezer.

6. Conclusion

Tregs as a cellular clinical therapy are rapidly developing after initial promising results in clinical trials. The procedure could even more evolve if Tregs could be efficiently cryopreserved and stored for future infusion or expansion rather than utilization of only freshly isolated and expanded cells as it is preferred now. Currently, knowledge regarding impact of cryopreservation on Treg recovery, viability, and functionality is still limited. Based on experience with PBMCs, cryopreservation may have a detrimental influence on Tregs and can: decrease Treg viability, cause abnormal cytokine secretion, and alter the expression of surface markers essential for proper Treg function and migration. Therefore, an optimized strategy for Treg cryopreservation in conjunction with culture, cell expansion, and processing for clinical application still needs to be investigated and defined.

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