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APPLICATION NOTE No. 453

Optimizing CD4+ T Cells Long-term Expansion Process in the DASbox® Mini Bioreactor System: Impact of the Dissolved Oxygen

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Abstract

T cell lymphocytes play a central role in the adaptive immune response. They are an essential tool of adoptive cell therapy for the treatment of chronic viral infections and malignant diseases. However, the development of cell-based therapy products generally requires the production of a large quantity of high-quality viable T cells in a controlled environment. Stirred-tank bioreactors can offer a suitable environment for the culture of T cells by providing homogeneous distribution of nutrients and gases, along with the maintenance of cells and molecules in suspension with a high process control capability. In this study, we tested the suitability of BioBLU® 0.3c Single-Use Bioreactors controlled by a DASbox Mini Bioreactor System in the long-term expansion of CD4+ T cells as well as the impact of different oxygen tensions on cell proliferation. The control of several growth parameters during cell expansion resulted in an efficient proliferation of highly viable and functional CD4+ T cells after 16 days of culture. Given that 100% dissolved oxygen (DO) in the bioreactor corresponds to 20.9% atmospheric oxygen level, incubation at two different oxygen tensions (70% or 20% DO) was carried out. The results suggested a tendency towards a positive impact of lower DO levels on CD4+ T cell proliferation rates. These results demonstrate the substantial potential of the DASbox Mini Bioreactor System used in combination with BioBLU 0.3c Single-Use Bioreactors to optimize T cell culture conditions.

Introduction

Adoptive cell therapy (ACT), a branch of immunotherapy, offers a promising treatment for chronic viral infections and malignant diseases such as cancer [1]. This innovative approach involves the autologous or allogenic transplant of immune cells into the patient's body. Specifically, T cells are extracted from the patient, modified genetically (if necessary), expanded *ex vivo* and reinfused into the patient to target viral or tumor antigens [2,3].

In clinical trials of various cancer treatments, adoptive cell therapy with tumor infiltrating lymphocytes (TIL), gene-modified T cells expressing novel T cell receptors (TCR), or chimeric antigen receptors (CAR) have shown favorable results in the eradication of different types of tumors. Other therapies in development include Cytokine-Induced Killer (CIK) cells, γδ T cells, Regulatory T (Treg) cells, and Natural Killer (NK) cells [4]. At this time, five adoptive cell

therapies have received approval by the US Food and Drug administration (FDA), all of them involving CAR T cells [5]. Such investigations have established that CAR T cell therapy is an important advance for children and young adults as well as for adult patients with resistant therapies, relapsed leukemia or other hematological malignancies [6].

In order to achieve successful therapeutic responses, the development of efficient and high-quality cell therapy products has become a challenge for clinical researchers and health care providers. As is true for every multistep process, the cell-based production workflow presents challenges of variability, complexity and other unresolved issues. Optimized protocols for the scalable manufacture of T cells are crucial, since T cells and other lymphocytes require meticulous culturing. They are highly sensitive to their culture environment and react easily by modifying their receptor/ligand repertoire, which can cause changes in cellular response to external substances and surfaces [7,8]. Additionally, T cells undergo frequent metabolic changes depending on the stimulation agents used for their activation *in vitro* [4]. They can enter into a quiescence or active state, move into the cell division cycle, go to apoptosis or differentiate [9]. Furthermore, due to the physiological and vascular structure of the bone marrow and lymphoid organs, immune cells experience a substantial gradient of oxygen tension *in vivo*, with oxygen concentrations reaching from 0.2 to 3% in the thymus and 0.5 to 4.5% in spleen and lymph nodes to 13% in the arterial blood [10-12]. Several studies have demonstrated that culture of T cells at different oxygen tensions (atmospheric (20%), physiological (2-12%) or hypoxic (< 2%) oxygen levels) can have a significant impact on cell proliferation, redox status and apoptosis activation [10,13-15]. Because of these complex characteristics, any small change in the culture environment can have profound consequences for the product quality.

One goal of preclinical research and process development is the determination of optimal cell culture conditions to achieve high-quality cells. Another key step towards the optimization of cell-based therapies is the production of sufficient cell quantities in a rapid time frame.

To this end, the use of 3D culture systems, such as stirred-tank bioreactors, presents several advantages compared to other culture systems, such as static culture in bags or flasks [4]. Bioreactors combine efficient mass transfer of oxygen and nutrients with high reliability of bioproduction and scalability. These advantages are ascribed to the accurate control of various critical parameters such as nutrient feeding, temperature-, DO-, pH-control, gas sparging, and agitation. In the present study, we used a DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors to evaluate the impact of different oxygen availabilities on T cell expansion efficiency.

Expansion of human $CD4^+$ T Cells was examined at two different oxygen tensions (70% or 20% of dissolved oxygen), given that 100% DO corresponds to 20.9% atmospheric oxygen level. This study shows that parallel bioreactor control systems are useful tools to optimize culture conditions before the scaling-up process, thus providing a solid basis for optimal yields in subsequent steps from benchtop to production scale.

Material and Methods

Culture of $CD4^+$ T cells in BioBLU 0.3c Single-Use Bioreactors

Lonza® human peripheral blood $CD4^+$ T cells (Lonza, 2W-200) were expanded on T75 CellBIND® flasks (Corning®, 3290). At day 0, cells were seeded at 1 x 10^6 cells/ml and activated with ImmunoCult® Human CD3/CD28/CD2 T Cell Activator (StemCell Technologies®, 10990) in ImmunoCult-XF Cell expansion medium (StemCell Technologies, 10981) supplemented with recombinant human interleukin 2 (rhIL-2) (StemCell Technologies, 78036). The cells were incubated at 37 °C, 5% CO_2 and 20% O_2 in a CellXpert® C170i Incubator (Eppendorf, 6731). Three days later, cells were counted using the Vi-CELL® automated cell counting device (Beckman Coulter®, 731050) and the culture volume was increased to adjust the viable cell density to ~ 1.0 to 2.5 x 10^5 cells/ml. After 5 days, cells were transferred to the bioreactor (DASbox Mini Bioreactor System, equipped with four BioBLU 0.3c Single-Use Bioreactors) at a cell density of ~ 1.0 to 3.0 x 10^5 cells/ml. Two protocols were evaluated in this study: in the first , the dissolved oxygen (DO) level was

set to 70%, while in the second the DO was set to 20%. All cultures were incubated at 37 °C. The agitation speed was adjusted to 70 rpm and the pH of the growth medium was maintained at 7.4 by automatic addition of CO_2 in the vessel headspace or addition of NaOH (1N) to the medium as summarized in Table 1. The culture was maintained for 21 days (5 days in T75 flasks + 16 days in the bioreactor) and the cell density was regularly adjusted according to the culture medium manufacturer's protocol by addition of fresh ImmunoCult-XF Cell expansion medium supplemented with rhIL-2 and partial harvesting. On day 7 and 14, the cells were re-activated with the ImmunoCult Human CD3/CD28/CD2 T Cell Activator in ImmunoCult-XF Cell expansion medium supplemented with rhIL-2 (Figure 1). Cell proliferation was evaluated at different time points during the cultivation with the Vi-CELL automated cell counting device. The lactate and glucose concentrations were monitored at different timepoints with the YSI® 2900 Biochemistry analyzer (YSI). The present study is based on three independent experiments with two different T cell donors.

Table 1: Overview of process parameters for the cultivation of $CD4^+$ T cells in BioBLU 0.3c Single-Use Bioreactors based on the protocol of Ou J. et al. [16].

Initial working volume	100 mL
Inoculation cell density (day 5)	1.0-3.0 × 10^5 cells/mL
Temperature	37 °C
pH	7.4
Agitation	70 rpm
Gas sparging rate	0.01 VVM
Dissolved oxygen (DO)	20 or 70 %

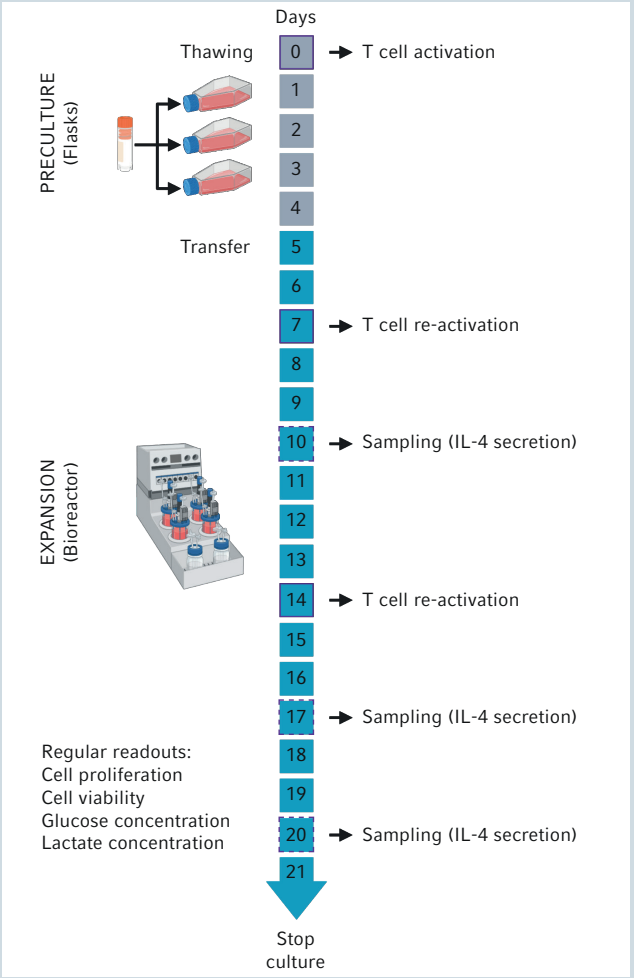

Fig. 1: Schematic representation of $CD4^+$ T cell expansion in BioBLU 0.3c Single-Use Bioreactors controlled by a DASbox Mini Bioreactor System for 21 days with a preculture of 5 days in flasks. Created with BioRender.com

To know more about the possibilities of configuring the DASbox system tailored to your needs, visit **www.eppendorf.group/dasbox**

Interleukin (IL)-4 production by $CD4^+$ T cells

On day 10, 17 and 20, cell culture samples were collected and seeded at a density of 1 x 10^6 cells/ml in a 24-well plate. $CD4^+$ T cells were stimulated with ionomycin at 2.5 µM (StemCell Technologies, 73722) and phorbol 12 -myristate 13 -acetate (PMA) at 10 ng/ml (StemCell Technologies, 74042) and incubated at 37 °C for 24 hours. At the end of the activation step, the cell suspension was collected and centrifuged at 200 x g for 5 min. The production of IL-4 by the $CD4^+$ T cells was measured in the clarified supernatant using the Invitrogen® IL-4 Human ELISA kit (Thermo Fisher Scientific®, BMS-225-2). Briefly, a combination of 50 µl of sample and 50 µl of biotin-conjugate anti-human IL-4 antibody was added to the anti-human IL-4 antibody coated plate and incubated at room temperature (RT) for 2 hours on a microplate shaker. At the end of the incubation, the plate was washed three times before the addition of 100 µl of diluted Streptavidin-HRP and incubated for 1 hour at RT. A total of 3 washes were also performed before the addition of 100 µl of the TMB substrate solution. The plate was incubated for 10 minutes at RT avoiding direct exposure to intense light before the addition of the stop buffer. Optical densities were recorded at 450 nm as a primary wavelength and 620 nm as the reference wavelength.

Results

CD4+ T cell expansion in BioBLU 0.3c Single-Use Bioreactors

Initially, CD4+ T cells were subjected to a static cultivation period of 5 days on T75-flasks to ensure appropriate cell numbers for the culture in the bioreactor. Next, the cells were seeded into two bioreactors at a density of ~ 1.0 to 3.0 x 10^5 cells/ml (~ 1.0 to 3.0 x 10^7 cells/vessel) and incubated at two different DO concentrations. In order to emulate the physiological oxygen levels that lymphocytes experience *in vivo* [10,17], DO in one culture was set to 20% oxygen saturation level whereas in the second protocol, the oxygen saturation level was set to 70%.

The culture was maintained in the bioreactor for 16 days (from day 5 to day 21). Cell density was adjusted every one to three days to maintain CD4+ T cells at lower cell density and improve cell proliferation and viability. Cell viability at each dilution point was comparable and remained stable under both conditions (between 97 to 87%) over the entire course of the experiment. Furthermore, high cell proliferation rates were achieved for both DO levels

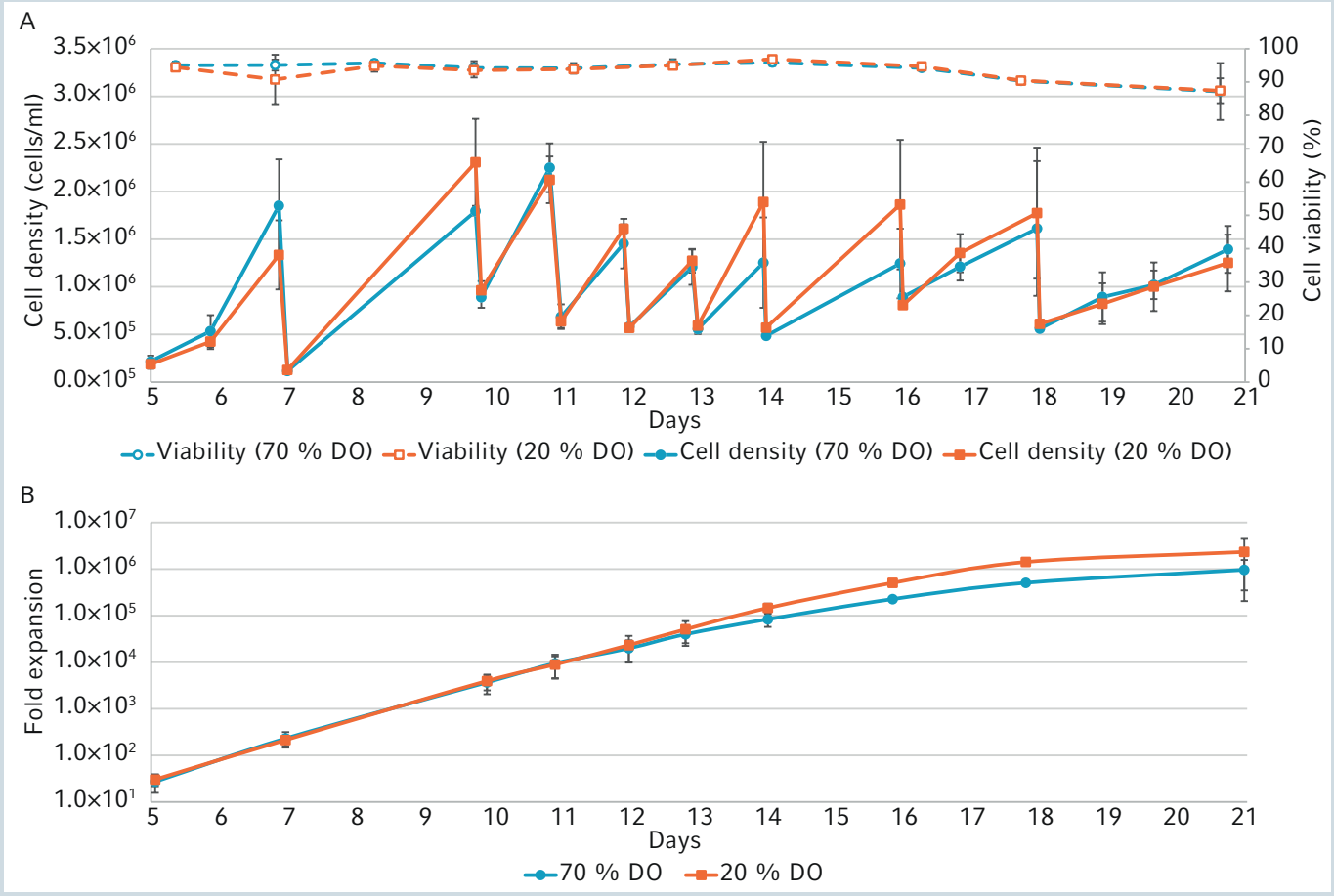

Fig. 2: CD4+ T cell proliferation in the BioBLU 0.3c Single-Use Bioreactor controlled by a DASbox Mini Bioreactor System during a 16 day-expansion phase (from day 5 to day 21). (A) Cell density and viability of CD4+ T cells under both conditions (70 or 20% DO) were monitored at different time points and cell density was adjusted accordingly to the manufacturer's protocol. (B) Average fold expansion of CD4+ T cultured at 70 or 20% DO. Results are compiled from three independent culture replicates.

during the incubation period. Taking into account the cell expansion in flasks prior to bioreactor inoculation (from day 0 to day 5) and the cell expansion in the bioreactor (from day 5 to day 21), the average fold-expansion of $CD4^+$ T cells cultured at 70% DO and 20% DO was of 9.63 x 10^5-fold and 2.35 x 10^6-fold on day 21, respectively (Figure 2B). These results indicate a possible positive impact of 20% DO on $CD4^+$ T cell numbers compared to 70% DO. This tendency is supported by a similar bioreactor study by Bohnenkamp *et al.* [18] which identified enhanced T cell expansion at lower air saturation (25% and 50% over 75%). Moreover, in a static study (using T-flaks) Carswell *et al.* [14] found increased proliferation rates of stimulated T cells grown at 5% atmospheric O_2 levels compared to cells grown at 20% atmospheric O_2 level.

In addition to growth factors, the proliferation of T cells in an *in vitro* environment is also regulated by substrate and metabolite concentrations. Metabolite monitoring during the whole culture process revealed comparable glucose consumption under both conditions (Figure 3). Cell density adjustment every 1 to 3 days by dilution allowed maintaining glucose levels within a narrow range, avoiding large concentration fluctuations that could adversely affect cellular metabolism.

Additionally, lactate production was monitored at each dilution point. As expected, lactate levels increased as the available glucose in the culture was consumed. However, as previously observed, cell density adjustment reduced fluctuation of lactate levels during the culture process. The fine control of these parameters is essential to maintain cells in a robust state and improve their proliferation.

$CD4^+$ T cell quality assessment by Interleukin (IL)-4 production

To ensure a high-quality product, a series of validation steps are required to establish the maintenance of T cell quality. In the present study the functional capability of $CD4^+$ T cells was evaluated by measuring the production of interleukin 4 (IL-4). This signature cytokine is involved in several immunological processes. It promotes the differentiation of

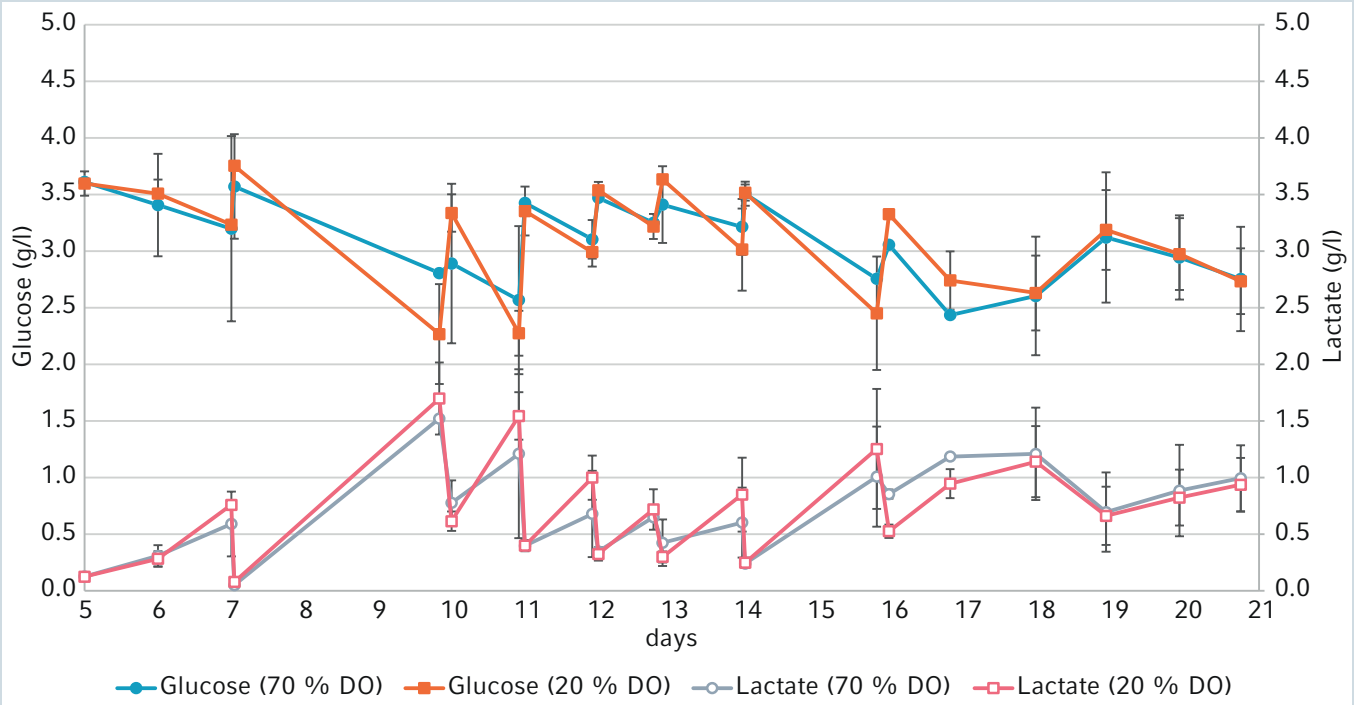

Fig. 3: Glucose consumption and lactate production profile of $CD4^+$ T cells cultured for 16 days in the BioBLU 0.3c Single-Use Bioreactor controlled by a DASbox Mini Bioreactor System. Results are an average of three independent culture replicates.

Th2 cells, a subset of T cells, it regulates immunoglobulin (Ig) class switch to IgG1 and IgE in the antibody response of B cells and induces alternative activation of macrophages in concurrence with IL-13 [19].

Bioreactor cultures were sampled at different time points (day 10, 17 and 20) and T cells were either left untreated (NS) or stimulated (ST) with PMA and ionomycin for 24 hours. Thereafter, the cell suspension was collected and centrifuged and the levels of secreted IL-4 were determined (see Material and Methods). As shown in figure 4, IL-4 inducibility was maintained in stimulated but not non-stimulated cells.

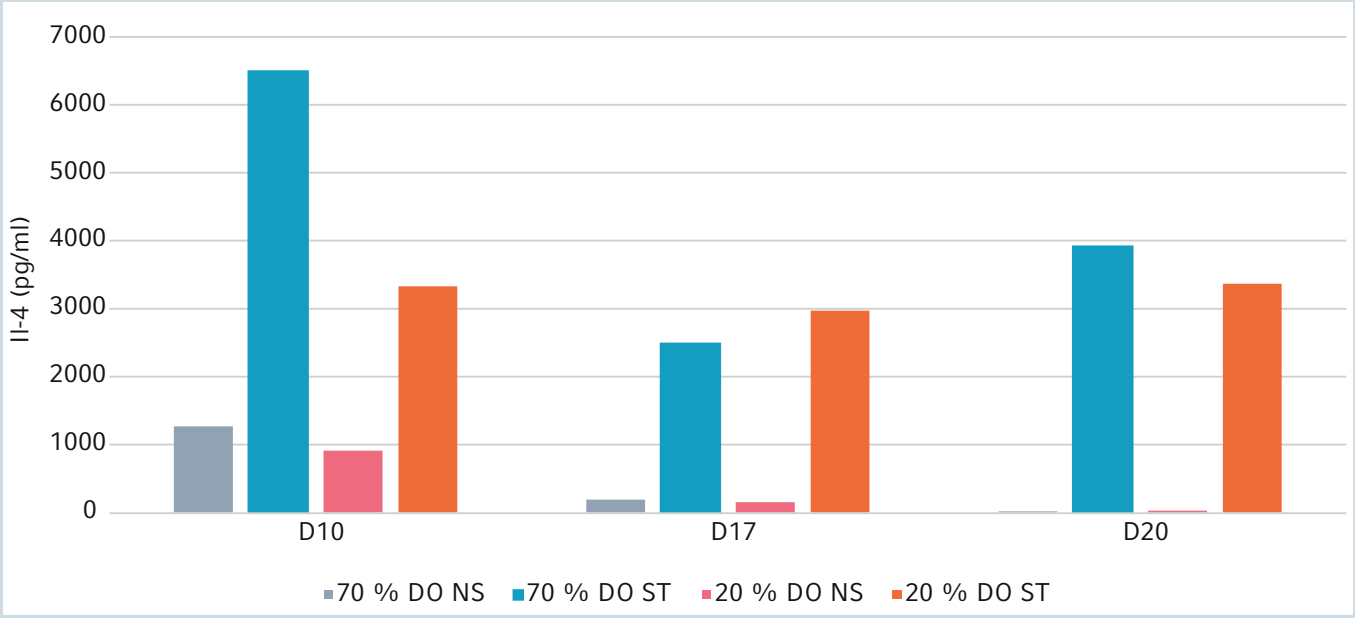

Fig. 4: Determination of Interleukin (IL)-4 production by $CD4^+$ T Cells cultured at 70 or 20% DO. Culture samples were harvested at day 10, 17 and 20 and seeded at a density of 1 x10^6 cells/ml in a 24-well plate. IL-4 production of stimulated (PMA and Ionomycin treatment for 24 hours at 37 °C) (ST) or non-stimulated (NS) cells, was assessed in the supernatant using an ELISA assay. Representative results are expressed in pg/ml of IL-4.

Conclusion

The importance of controlling oxygen levels in T cell culture has been emphasized in several studies [4, 9, 10, 16, 18]. However, culturing methods (flasks, bags or bioreactors) and the level of fine tuning control of the critical parameters (temperature, pH, DO, etc.) differs from one study to another, which may lead to disparate conclusions reached in the current literature [13,14, 20]. The DASbox Mini Bioreactor System offers a fully controllable culture system appropriate to generate high-quality viable and functional T cells throughout the entire process, which will impact the quality of the data. The present study illustrates the successful long-term expansion of $CD4^+$T cells in a DASbox Mini Bioreactor System equipped with BioBLU 0.3 Single-Use Bioreactors. A possible positive impact of a lower oxygen tension (20% instead of 70% DO level) was suggested on $CD4^+$ T cell proliferation rates without impacting cell functionality

in terms of IL-4 secretion. Finally, reproducibility, a key requirement for the optimization of scalable bioprocessing, was confirmed by testing T cells from different donors. Thus, the approach presented here illustrates the advantages of a controllable bioreactor-based culturing systems for the development of scalable T Cell production processes.

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Ordering information

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AA453STS020-01/062022