A System Approach to Improving Yields in a Disposable Bioreactor

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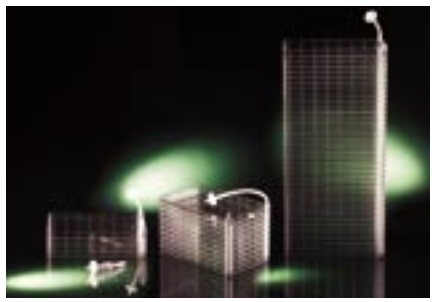
In 1978, scientists in the biotech department of Rentschler Biotechnologie GmbH (Germany) described a multitray cell-culture system for producing interferon beta with adherently growing human fibroblasts. The researchers wanted to improve production efficiencies from the then-current practice of using T-flasks by providing larger surface areas and reducing handling efforts. At the time, the process involved opening and closing 600 T-flasks per day. During interferon induction, each bottle had to be opened 11 times for changing media, washing with buffers, and adding different chemicals. In addition to the intensive resource allocation required to perform those tasks, the product was subject to a heightened risk of contamination each time a bottle was opened. A closed multitray system was developed in collaboration with scientists from Nunc in Denmark, and it ultimately came to market as the Cell Factory system from Nalge Nunc International. Gas exchange was a limiting factor for some cell types with this system, so Nunc developed active gassed Cell Factories.

Today, disposable or single-use technology is used in many stages of manufacturing biological products, primarily to eliminate cleaning validation and prevent contamination. Constant development of new technologies and improvement of existing technologies encourage biopharmaceutical manufacturers to review their production processes, both upstream and downstream, to look for potential efficiencies. Here, we summarize findings from two publications, one of which describes how gas exchange led to increased yields using a disposable bioreactor; the other uses the bioreactor in scaling up a dendritic cell process.



The NUNC Cell Factory system for active gassing incorporates a patented gas-flow system to enable a controlled atmosphere equally distributed through the culture trays. Users specify a gas mixture that is actively pumped through the premounted filter.
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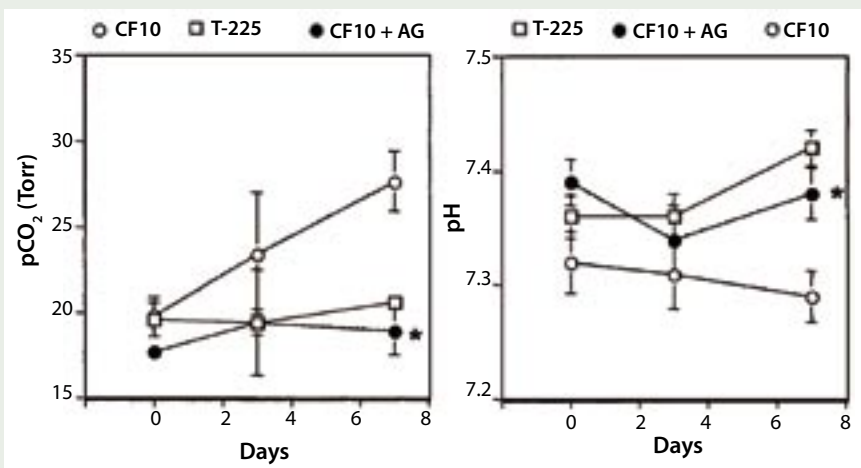
Improvements with Active Gassing

Active gassing describes gas exchange within a closed system that results in a consistently uniform distribution of gas. According to Okada et al. (2), active gassing significantly improved cell yield in a study that compared a 10-tray Cell Factory system, a 10-tray actively gassed Cell Factory system, and 28 actively gassed standard tissue-culture flasks in large-scale production of recombinant viruses. An earlier protocol for such large-scale production in large culture vessels was problematic because of inadequate gas exchange. But the active gassing capability successfully improved virus production on a large scale and enabled researchers to improve both labor- and cost-efficiency with a closed system.

Figure 1: CO_2 concentrations (LEFT) and pH (RIGHT) were determined in conditioned medium ($n = 4$) with and without active gassing (AG). A T-flask (T-225), Cell Factory system (CF10), and actively gassed Cell Factory system were used. Asterisk indicates $p < 0.05$ compared with the CF10 without AG.



Product Focus: All products of cell culture bioprocesses

Process Focus: Production

Who Should Read: Cell culture engineers, manufacturing, process development, QA/QC

Keywords: Disposables, process optimization, cell culture

Level: Intermediate

Table 1: Increased cell growth and vector yield with active CO_2 and air exchange; cell growth and vector yield per cell were compared in 28 flasks with a surface area of 225 cm^2 each (total surface area of 6300 cm^3) and a 10-tray Cell Factory system with a total surface area of 6320 cm^3.

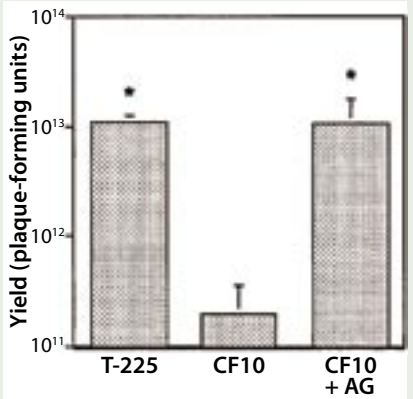
Cell Culture System	Number of Cells Harvested	Vector Yield (PFU*/cell)
225-cm^2 flasks **	$(1.4 \pm 0.2) \times 10^9$	7.9×10^3
CF10	$(4.9 \pm 1.6) \times 10^8$	4.1×10^2
CF10 + AG	$(1.3 \pm 0.3) \times 10^9$	8.2×10^3

* PFU = plaque forming units ** Per each of 28 flasks

Table 2: Enhanced gas exchange and maintenance of pH in conditioned medium after plasmid transfection; means ± standard deviations are shown ($n = 4$); CO_2 concentrations and pH in the CF (with or without AG) were estimated three days after plasmid transfection.

Cell Factory System	pCO_2	pH
CF10	25.6 ± 1.1	7.23 ± 0.03
CF10 + AG	14.2 ± 0.1	7.40 ± 0.01

Figure 2: The number of plaque-forming units (PFU) revealed that adenovirus vector production was improved by active gassing. The asterisk indicates $p < 0.05$ compared with a CF10 without AG.



Materials and Methods: To propagate the vectors used in the study (**3–5**), human embryonic kidney derived (HEK 293) cells were grown either in a flask with a surface area of 225 cm^2 (T-225 Falcon from BD Biosciences Discovery Labware in Bedford, MA; www.bd.com) or in a 10-tray Cell Factory system (CF10 from Nalge Nunc International of Rochester, NY, www.nalgenunc.com). Active gassing was achieved with a pump supplying 5% CO_2 to the CF10 at rate of 500 mL/min. To connect the pump, the CF10's gas inlet was mounted with a bacterial air filter from Pall Gelman Sciences of Ann Arbor, MI (www.pall.com). The CF10 was then placed inside an incubator, which provided humidified, controlled atmospheric circulation. Air flow of less than 200 mL/min provided an uneven distribution of gas, negatively affecting cell growth to great extent. A blood gas analyzer (Nova PHOX from Diamond Diagnostics of Holliston, MA, www.diamonddiagnostics.com) was used to measure CO_2 concentrations and pH, and glucose levels in the culture medium were estimated with a glucose meter.

Results and Discussion: Application of active gassing increased cell proliferation substantially in the CF10 (Table 1). After both adenovirus infection and AAV transfection, CO_2 concentrations in the media remained at their initial levels when active gassing was applied to both the T-225 and CF10 systems. Conversely, a lack of active gassing increased CO_2 in the CF10 for both the infection and transfection experiments (Figure 1 and Table 2). The culture medium pH was similar in both cell culture systems, but it was much lower in the CF10 that was not actively gassed (Figure 1).

When adenovirus vector yields were estimated using 28 T-225 flasks, a CF10 with a 6329-cm^2 surface area, or the actively gassed CF10, the latter exhibited yields that were 53.4× greater than those from the CF10 without active gassing (Figure 2). Active gassing significantly

Figure 3: FACS-stained surface markers CD1a, CD80, CD83, CD86, HLA-DR and HLA-ABC were used to phenotype both iDC (TOP) and mDC (BOTTOM). Mean ± standard deviation is shown; data were obtained from five independent experiments.

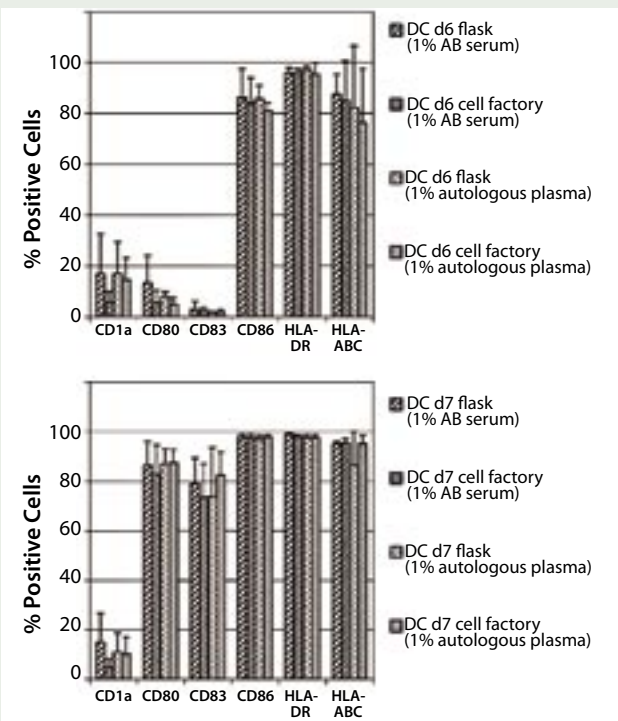


Figure 4: Immunophenotype was analyzed in both iDC and mDC 48 hours before (TOP) and after (BOTTOM) withdrawal of cytokines. The percentages of marker expression are shown in the top panel, and intensity of surface marker expression is shown in the bottom panel.

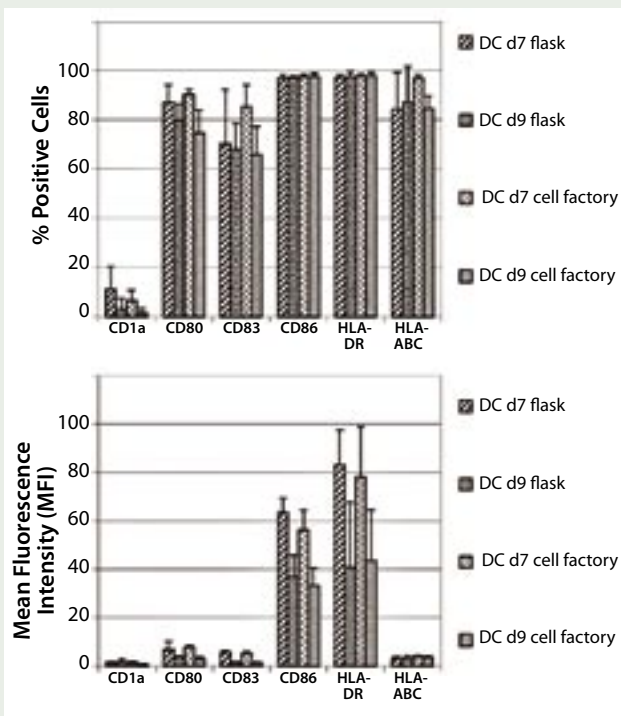


Table 3: Improved yields of recombinant AAV type 1 by active gas exchange; means ± standard deviation are shown ($n = 4$); AAV1-EGFP yields in CF10 were compared with and without active gassing.

Cell Factory System	Yield per Vessel	Yield per Cell
CF10	$(2.2 \pm 0.5) \times 10^{12}$	$(3.1 \pm 0.6) \times 10^{3}$
CF10 + AG	$(1.0 \pm 0.7) \times 10^{13}$	$(1.1 \pm 0.7) \times 10^{4}$

Nalge Nunc International single-use systems include containers, closures and tubing sets.
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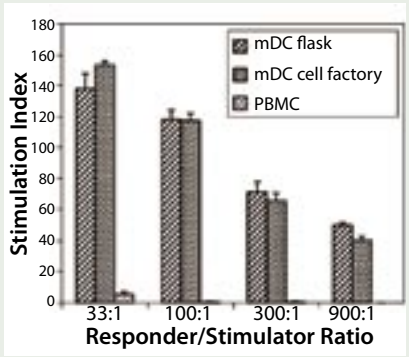
improved CF10 vector yields per producer cell for both the infection and transfection (Tables 1 and 3).

Implementation of large-scale cell culture vessels, CF10 and CF40, demonstrated comparable and improved yields when compared with tissue-culture flasks. Use of a disposable bioreactor that is both scalable and adjustable in production capability suggests cost- and labor-efficient scale-up for culturing adherent cells on a production scale. The active gassing system secures a controlled atmosphere in culture trays through equal distribution of a user-specified gas mix actively pumped through a premounted filter. It is suitable for culturing and nursing cells with controlled gas distribution, especially those oxygen-demanding and/or pH-sensitive cells that may benefit from a controlled atmosphere.

A Sterile, Closed System

The large-scale production capability of the Cell Factory system was further demonstrated in a study by Tuyaerts et al. (**6**). The authors compared research-grade dendritic cells (DC) produced in a standard tissue-culture flask (TCF) protocol (**7, 8**) with those produced in large numbers using Cell Factory systems. Large-scale

Figure 5: Graded doses of DC were added to 2×10^5 allogeneic CD4+ T lymphocytes for five days to test the allostimulatory capacity of mature DC. [^{3}H]Thymidine uptake was measured, and the stimulation index was calculated by subtracting the number of counts in a coculture by the counts of DC alone, then dividing that figure by the counts for T cells alone. Mean ± standard deviation is shown.



production suitable for use in human vaccine approaches also requires compliance with CGMP guidelines. Most principal research on DC was performed with cells generated from monocytes adhered to TCF. The authors developed a similar culture system on a larger scale that would generate "laboratory-grade" DC. Their comparisons involved phenotype, purity, and the capacity to internalize dextran, to secrete interleukin 12 (IL-12), to stimulate allogeneic T cells (CD4+), and to activate memory cells specific to influenza matrix protein (IMP1) after electroporation with IMP1 encoding mRNA (**9**).

Materials and Methods: To apply the original protocol for DC generation to the Cell Factory system, the researchers designed tubing sets with sterile connections and septa for injections. Those were necessary for transferring peripheral blood mononuclear cells (PBMC) into the culture, to perform necessary cell-washing steps, to harvest cells, and to add cytokines — all described in a protocol published by Romani et al.

Compared with conventionally produced DC in TCF, the Cell Factory system produced DC of equal quality or slightly better according to all testing. Immunophenotypic analysis (measuring the presence of surface markers CD1a, CD80, CD83, CD86, HLA-DR, and HLA-ABC) of both immature DC (iDC on Day 6, D6) and mature DC (mDC on Day 7, D7) showed little to no difference between the two (Figure 3).

To determine the phenotypic stability, all cytokines were removed from the cultures for 48 hours. A fluorescence analysis was then performed using a FACScalibur flow cytometer and monoclonal antibodies (CD14-FITC, CD80-PE, CD83-PE, CD86-PE, HLA-ABC-FITC, and biotinylated HLA-DR) in conjunction with Cell Quest software, all from BD Biosciences. Nonreactive isotype-matched antibodies (also from BD) were used as controls. The percentage of DC expressing the surface markers did not change, but the level of expression decreased (Figure 4). The mDC remained morphologically stable and viable during the stability test, which confirms that cells generated in both TCF or CF and matured in the inflammatory cytokine cocktail were fully mature and did not dedifferentiate to iDC or into macrophages.

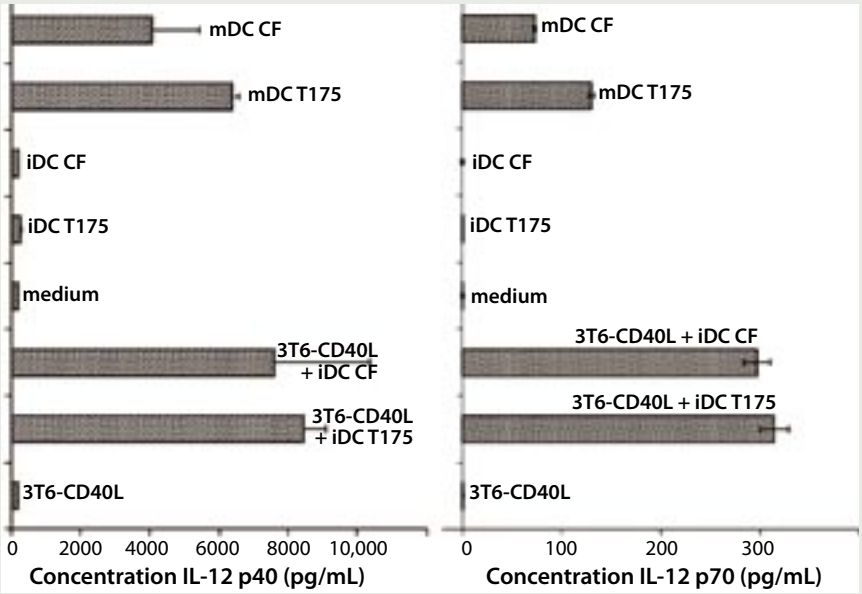
To test the mDC's capacity to stimulate allogeneic T cells, graded amounts of mDC (stimulators) were added to 2×10^5 allogeneic CD4+ T cells (responders) for five days to create four different ratios of responders and stimulators (R:S). Figure 5 shows no significant difference in the allostimulatory capacity between mDC-TCF and mDC-CF. To test for cytokine secretion, interleukin-12 (IL-12) secretion was measured through ELISA after a DC-to-CD40 (membrane-bound CD40L) ligation. Again, both TCF and CF showed comparable results, with IL-12 secreted in similar amounts (Figure 6).

Next, iDC-TCF and iDC-CF were tested for their endocytic and phagocytic

Table 4: Viability, purity, and recovery of frozen/thawed DC; 30 minutes after cryopreserved mDC were thawed, viability was assessed by exclusion of Trypan blue; purity was calculated using FSC/SSC characteristics of DC and T-cells in FACS; recovery of thawed DC was calculated as the percentage of viable DC recovered from the original number frozen; mean ± standard deviation are listed.

	mDC-TCF	mDC-CF
Viability mDC before freezing	93.75 ± 3	89 ± 7
Purity mDC before freezing	74.25 ± 16	90.5 ± 4
Viability mDC after thawing	90.33 ± 3	86 ± 14
Purity mDC after thawing	83 ± 8	91.25 ± 2
Recovery of mDC	94.67 ± 24	75.75 ± 28

Figure 6: IL-12 plays an integral role in the activation of antigen-specific, naive Th1 cells. IL-12 secretion by DC was tested by coculturing with CD40L-expressing cells at a ratio of 1:1 for 48 hours, then screening for IL-12 p40 (TOP) and IL-12 p70 (BOTTOM). Mean ± standard deviation is shown.



capabilities. These relate to the capture and processing of antigens, as well as the presentation of antigenic epitopes to T cells, which represent a major function of DC. FITC-conjugated dextran was used to test the receptor-mediated endocytic capabilities of iDC-TCF and iDC-CF, and FITC-conjugated latex beads were used to test phagocytosis. Capacities were measured as percentages of CD86-expressing cells that had taken up the dextran-FITC and latex-bead–FITC molecules, respectively. No difference was observed between iDC grown in TCF and those grown in CF.

Because cryopreservation was required for DC use in clinical trials, the viability, purity, and recuperation of frozen cells were then tested (**10–16**). Mature DC were frozen at a concentration of 6–10 × 10^6 cells/mL in human AB serum or autologous plasma with 10% DMSO and 2% glucose for one to four months. Then they were thawed and assessed for viability after 30 minutes by exclusion of Trypan blue. Cell purity was calculated by the FSC–SSC (forward scatter and side scatter) characteristics of DC and lymphocytes in a flow cytometer. DC recovery was measured as the percentage of original cells that remained viable after thawing. Phenotypic changes in the thawed DC were detected by flow cytometry (Figure 7). No changes in viability or surface-marker expression were observed in fresh and thawed mDC. These results confirmed that mDC generated in TCF or CF could be frozen and thawed without loss of viability, purity, or surface-marker expression.

Figure 7: After thawed DC were put back into culture for 24 hr, phenotypic analysis was performed using flow cytometry.

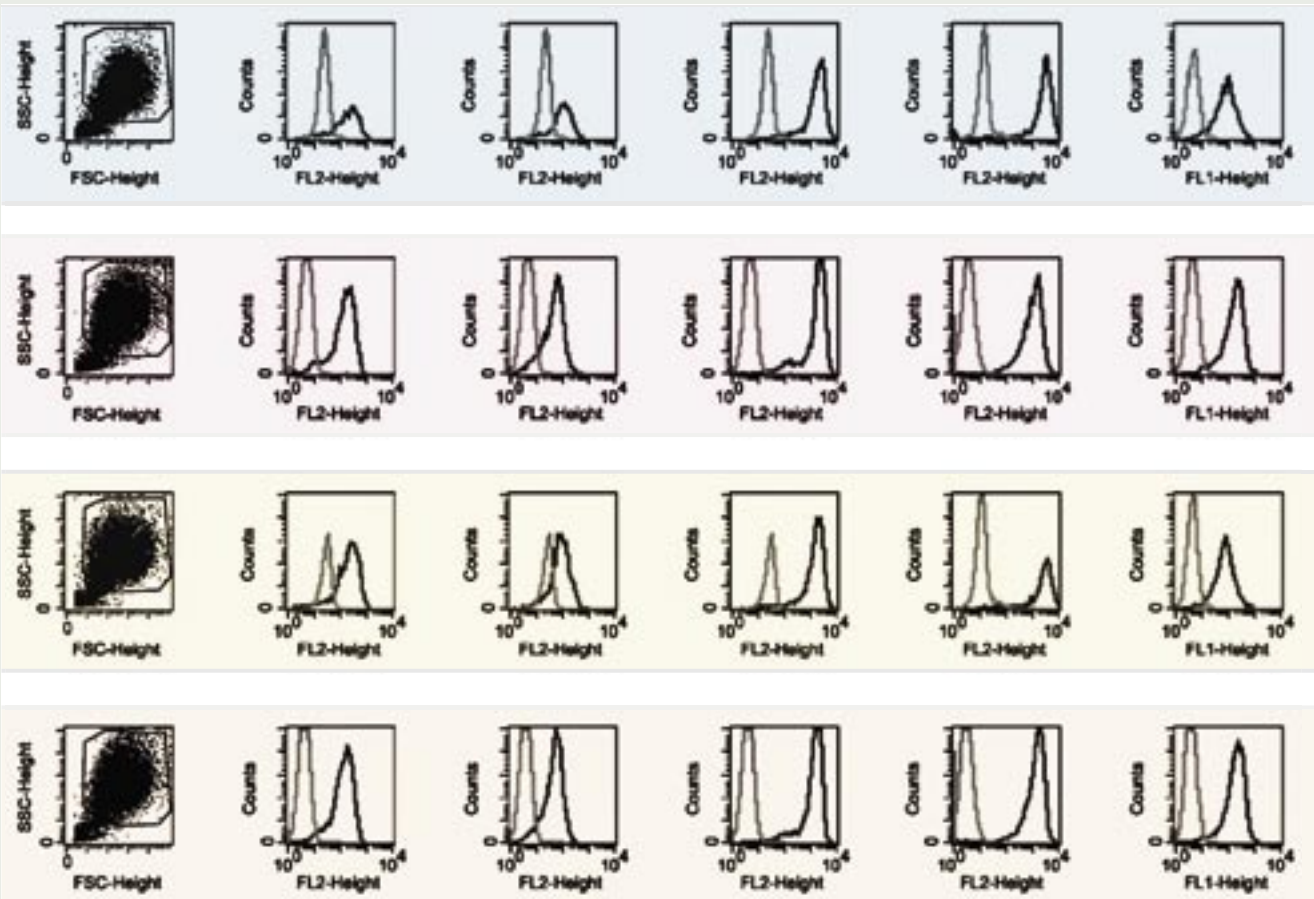
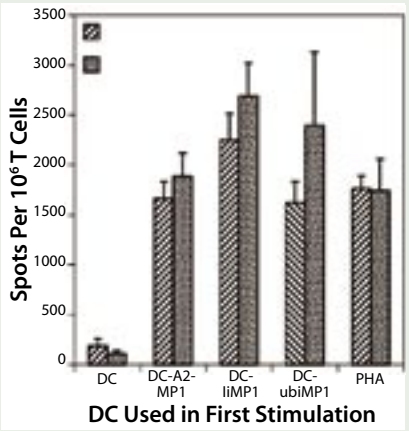


Figure 8: Graded DC doses were added to 2×10^5 allogeneic CD4+ T lymphocytes for five days to test activation of memory T cells by DC electroporated with influenza matrix protein 1 (IMP1) encoding mRNA. First, IMP1 expressing mDC were cocultured with autologous CD8+ T cells for seven days. Then, IFN-γ secretion was measured to test for T cell (10^6) activation in ELISPOT plates.



In further consideration for clinical trials, an additional experiment was conducted to compare DC produced in both systems for their potential to induce an antigen-specific immune response through memory cells (CD8+ T cells) in vitro. First, mRNA encoded with IMP1 was transfected into DC using a highly efficient electroporation procedure (**9, 16**). Then the IMP1-expressing mDC were cocultured with autologous CD8+ T cells for seven days. After that incubation period, activated T cells were restimulated overnight in ELISPOT plates with the IMP1-electroporated DC to detect and measure their resulting IFN-γ secretion. The DC generated in CF were found to be as potent in inducing an influenza-specific response as those generated in TCF (Figure 8).

Results and Discussion: This study demonstrated that DC can be generated in large quantities from peripheral-blood mononuclear cells (PBMCs) enriched by adhesion to CF in a fully closed system. Furthermore, dendritic cells produced in a Cell Factory system were proven to be functionally equivalent to dendritic cells produced in a conventional tissue culture flask system, which was an important study objective. The results concluded that DC generated in a CF system and electroporated with mRNA encoding tumor antigen can be used as clinical-grade vaccine for cancer patients.

A Holistic Approach

These two studies have demonstrated the flexibility of a disposable bioreactor system, as well as some of the dynamics introduced within such a closed system at large scale. Implementing a closed system at production scale introduces process dynamics that may be addressed by a holistic approach to process design. A vendor capable of assisting in all aspects of building a disposable cell culture system, including those of fluid transfer and containment, provides much-needed support in this burgeoning field. In both of the studies described here, improvements to a closed system improved product yields. The need for a holistic approach to implementing a disposable bioreactor system with a consultative vendor capable of both upstream and downstream bioprocessing equipment solutions is clear.

Development of this large-scale cell culture system continues to improve yields, as described in the examples presented here. However, a disposable bioreactor system involves more than its cell culture vessel alone. Tubing and containment devices are integral to maintaining sterile techniques in a closed system. Scientists should consider all fluid-containment and transfer devices as part of a bioproduction suite and seek coordinated development of those items as part of an overall solution.

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