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HEMATOPOIETIC STEM CELLS

Automated CD34+ cell isolation of peripheral blood stem cell apheresis product

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Abstract

Background aims. Immunomagnetic enrichment of CD34+ hematopoietic "stem" cells (HSCs) using paramagnetic nanobead coupled CD34 antibody and immunomagnetic extraction with the CliniMACS plus system is the standard approach to generating T-cell-depleted stem cell grafts. Their clinical beneficence in selected indications is established. Even though CD34+ selected grafts are typically given in the context of a severely immunosuppressive conditioning with anti-thymocyte globulin or similar, the degree of T-cell depletion appears to affect clinical outcomes and thus in addition to CD34 cell recovery, the degree of T-cell depletion critically describes process quality. An automatic immunomagnetic cell processing system, CliniMACS Prodigy, including a protocol for fully automatic CD34+ cell selection from apheresis products, was recently developed. We performed a formal process validation to support submission of the protocol for CE release, a prerequisite for clinical use of Prodigy CD34+ products. *Methods.* Granulocyte-colony stimulating factor–mobilized healthy-donor apheresis products were subjected to CD34+ cell selection using Prodigy with clinical reagents and consumables and advanced beta versions of the CD34 selection software. Target and non-target cells were enumerated using sensitive flow cytometry platforms. *Results.* Nine successful clinical-scale CD34+ cell selections were performed. Beyond setup, no operator intervention was required. Prodigy recovered 74 ± 13% of target cells with a viability of 99.9 ± 0.05%. Per 5 × 10E6 CD34+ cells, which we consider a per-kilogram dose of HSCs, products contained 17 ± 3 × 10E3 T cells and 78 ± 22 × 10E3 B cells. *Conclusions.* The process for CD34 selection with Prodigy is robust and labor-saving but not time-saving. Compared with clinical CD34+ selected products concurrently generated with the predecessor technology, product properties, importantly including CD34+ cell recovery and T-cell contents, were not significantly different. The automatic system is suitable for routine clinical application.

Key Words: *allogeneic, automation, cell therapy, clean room, CliniMACS, CD34, good manufacturing practice, haplo-identical, immunomagnetic, naked haplo, Prodigy, stem cell transplantation*

Introduction

CD34+ selected allogeneic stem cell grafts are used for patients at very high risk of severe graft-versus-host disease because of poor human leukocyte antigen matching (specifically in the haplo-identical setting), patients intolerant to immunosuppressants or those with non-malignant diseases who will not benefit from immunological graft-versus-leukemia effects [1–5]. CD34+ selected autologous grafts are being used for stem cell support for autoimmune disease and some pediatric solid tumors [6–11]. Manufacturing of CD34+ selected grafts with the CliniMACS Plus system is approved in many countries [12]. The selection package currently consists of a semi-automatic immunomagnetic selection/depletion device, CliniMACS Plus, and cognate

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selection software, as well as nanobead-coupled antibody (AB) and a single-use tubing system with magnetic column. The protocol consists of successive platelet depletion, AB incubation, magnetic selection and column elution/formulation. The T-cell depletion achieved with this method is so profound that very sensitive flow cytometry protocols are required for residual T-cell enumeration. Although semi-manual and complex, the process is of sufficient robustness to consistently generate clinical products for transplantation. Labor consumption is considerable, almost 4 hours, more than 3 hours of which require the presence of two operators. Recently, a protocol was introduced for the automatic cell manipulator CliniMACS Prodigy, which shares important components with CliniMACS Plus and in principle performs the same protocol as CliniMACS Plus, but fully automatically. This protocol was validated with research mobilized apheresis products under full-scale clinical conditions to generate data for submission for regulatory approval/CE marking. Data from this validation are presented and compared against data from CliniMACS Plus products, which were concurrently generated by the same operators and the quality of which was assessed with the same flow cytometry platform.

Methods

Donors and cells

Granulocyte-colony stimulating factor–mobilized apheresis products were collected from healthy volunteers who had undergone apheresis for unrelated donor stem cell donation [13] and had agreed to extend the apheresis for the purposes of this validation. Written informed donor consent was collected. The study was performed in agreement with the Helsinki declaration with ethics committee approval (ethics committee of Johann Wolfgang Goethe University Medical Center, Frankfurt, protocol #486/13). Aphereses were performed with standard Terumo mononuclear cell apheresis equipment as previously described [14,15], not to exceed a total apheresis duration (clinical graft plus validation product) of 300 min. Target hematocrit was <4 mg/dL. Apheresis products containing 4.75 ± 0.5 × 10E10 (mean ± SEM; range: 2.3–7.2 × 10E10) white blood cells with 4.8 ± 0.6 × 10E8 (mean ± SEM; range: 2.3–7.6 × 10E8) CD34+ cells and 38 ± 5% (mean × SEM; range: 7–51%) neutrophil content were used. Apheresis collections and selections were done in the second quarter of 2014.

Hemocytometry

Leukocyte concentrations in starting population, non-target population and target population were determined using the Sysmex XT1800 automatic hemocytometer for plausibility control against the single-platform flow cytometry assays. Flow cytometry was performed with FACSCalibur and LSRFortessa (Becton-Dickinson). Cells were stained with 7-AAD viability dye (BD Biosciences) and the following antibodies (all from BD Biosciences unless otherwise noted): anti-CD45-FITC (2D1)/anti-CD34-PE (8G12) (BD Stem Cell Reagent), anti-CD14-V450 (MφP9), anti-CD3-APC (SK7), anti-CD4-AmCyan (SK3), anti-CD8-APC-Vio770 (BW135/80, Miltenyi Biotec), anti-CD20-APC-eFluor780 (2H7, eBioscience) and anti-CD56-PE-Cy7 (CMSSB, eBioscience). *In-vitro* diagnostic-grade ABs were used where possible. Three platforms each were tested on apheresis product, positive and negative fraction (the commercial single-platform stem cell enumeration (SCE) kit; BD Biosciences), our clinical routine single-platform residual T-cell detection panel and a second residual cell identification panel designed for extended characterization of non-CD34+ cells for the purpose of these studies. The first two platforms were formally validated, are described in standard operating procedures and were performed in accordance with those procedures; the residual T-cell detection panel, in addition to containing BD counting beads for single-platform T-cell enumeration, includes CD34 and CD45 AB and ISHAGE (International Society for Hematology and Graft Engineering, now ISCT, International Society for Cellular Therapy)-conforming gating for bona fide CD34+ cells, to allow for additional cross-validation/plausibility control against the robust and simple three-color SCE protocol [16]. The extended research panel, including additional quantification of CD20 and CD56 and ISHAGE-conforming gating for CD34+ cell enumeration, was not formally validated to GMP level, but results were similarly compared against the two other, formally validated panels. Given the demonstrable precision of CD34+ cell enumeration, whenever the CD34+ cell count with any of the residual cell panels differed from the count with SCE by ±10%, the measurement had to be repeated. Unless otherwise indicated, all cell concentrations, frequencies or numbers refer to 7AAD-negative (viable) cells only.

Selection reagents and consumables

The CliniMACS Prodigy device [17,18], Prodigy TS310 tubing sets, CliniMACS CD34 Reagent (2 vials) and CliniMACS PBS/EDTA buffer were received from Miltenyi Biotec. NaCl 0.9%, H_2O injectionem and human serum albumin (HSA) were from Baxter.

CD34 selection process

The principle of immunomagnetic CD34 selection with nanobead-conjugated CD34 antibodies was first described 20 years ago by Shpall and colleagues [19]. Of the multitude of competing methods available at the time, only the Miltenyi system continues to be in use today [6,20]. In principle, the process remains unchanged, although a progression of increasingly user-friendly devices for the extraction of immunomagnetically labeled cells have become available. CliniMACS Prodigy is the most recent device in this succession. Magnet and reagent being the same as for the predecessor technology, the same apheresis product specifications (upper limits for total white blood cells [1.2 × 10E11] or total CD34+ cells [1.2 × 10E9] per two vials of reagent and TS310 tubing set as used in this validation) are applied to Prodigy and CliniMACS Plus. In these studies, because the small-scale (one reagent) separation protocol is not yet available, for all selections, two vials of CD34 reagent were used; that is, CD34 selection reagent was slightly "overdosed." After booting the Prodigy device, the CD34 selection protocol is selected, and the tubing set installed as directed by the user interface. Liquid reagents, including two vials of CD34 reagent, and apheresis product are connected as directed. An advanced beta version of the large-scale CD34 selection software was used; the CD34 selection package on Prodigy is currently not yet CE-marked (CE mark is expected in the second quarter of 2015 on the basis of the data presented here), and thus none of the cell products were intended for clinical use. Without making relevant changes to the established process for CliniMACS Plus [12], Prodigy successively and fully automatically performs platelet washes, CD34 AB-nanobead conjugate incubation, immunomagnetic column selection and cell elution. The specification of the allogeneic CD34-selected CliniMACS Plus product, for which our institution holds a marketing authorization, stipulates, in addition to being non-infectious with a panel of blood-transmissible agents as directed by the German Transfusion Act and bioburden-negative, a viable CD34+ cell dose in excess of 4 × 10E6/kg and a viable CD3+ cell dose below 5 × 10E4/kg of the recipient in at least 90% of products. B-cell content must be measured and declared. Shelf life is 72 h from the end of the apheresis, by which time the product must have been either infused or cryopreserved. Additional quality controls for the purposes of this validation include immunological differentiation of additional non-CD34+ cells (i.e., natural killer cells, monocytes and granulocytes), as well as assessment of product stability over time. CD34+ cell recovery and log-depletion of T cells are routinely calculated as measures of process quality. The goal of this validation exercise was to generate nine successive products meeting the regulator-approved specification of CD34-selected allogeneic stem cell products.

Statistics

Data were entered into Microsoft Excel spreadsheets from which descriptive statistics were extracted. Log T-cell depletion was calculated as the negative logarithm to base 10 of number of total T cells in the final product divided by the number of T cells in the apheresis product. Results from eight concurrently performed clinical CD34 selections on CliniMACS Plus and flow cytometrically analyzed with the same residual T-cell platform served as informal comparator; Student's *t*-test was used to identify statistically significant differences between CliniMACS Plus and Prodigy products; significance was assumed at $P < 0.05$.

Results

Process and process stability

In total, nine Prodigy runs were performed. Runs were initiated between 1 and 18 h after the end of the apheresis. Initial instrument setup with preparation and sterile connection of buffers and cells as well as installation of the tubing set were uneventful and took approximately 1 h of continuous operator hands-on time. Thereafter, the in total 5.75-h-long Prodigy process began, after which performance of quality control assays (described earlier) and product clearance can begin. Overnight processing without operators in attendance is in principle feasible, and some processes were performed in this fashion. Guidance during setup and operation by the touchscreen graphic user interface was flawless and easy to follow; kit installation and deinstallation required minimal training. Except that potentially the wrong fluids could be connected to the ports, which the machine would not detect, there is little room for handling errors and complex checks test kit installation and kit integrity. Process steps requiring operator intervention (typically in the presence of a second operator), operator supervision (one operator) or running automatically are drawn to a time axis in Figure 1; for comparison, the CliniMACS Plus process is depicted.

Product properties

Prodigy products contained 3.5 ± 0.5 × 10E8 (mean ± SEM) (range: 1.5–5.9 × 10E8) leukocytes, of which 3.4 ± 0.5 × 10E8 (mean ± SEM) (purity mean ± SEM: 93.9 ± 0.8%; range: 89.5–97.9%) were CD34+ cells and 9.4 ± 1.3 × 10E5 (mean ± SEM; range:

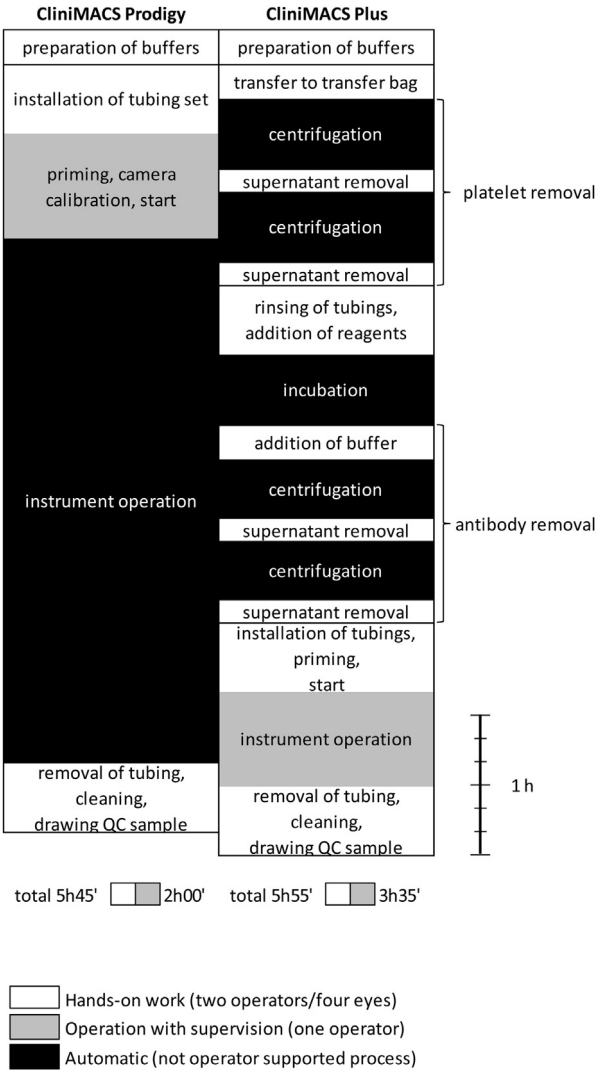

Figure 1. Comparative schedules of the CD34 selection process with CliniMACS Plus and CliniMACS Prodigy. White, gray and black bars: column height depicts time, shading marks manual, automatic-supervised and autonomous preparation steps, respectively. Process time is similar for both protocols, but hands-on time with Prodigy is much reduced.

5.4–18.5 × 10E5) were CD3+ cells, approximately 60% CD4+ and 40% CD8+. This reflected a recovery of 74 ± 13% (mean ± SEM; range: 49.3–100%) of CD34+ cells and a depletion of 99.99 ± 0.0% of T cells. Starting with a T-cell content of 24 ± 4 T cells per CD34+ cell (mean ± SEM), products had a CD34+ to T-cell ratio of 390 ± 67 (mean ± SEM; range: 144–830). The T-cell depletion achieved with Prodigy as calculated with the most frequently used formula (see Methods) was 4 log (mean ± SEM: 9.7 × 10E-5 ± 7.7 × 10E-6, range: 1.21 × 10E-4–6.6 × 10E-5). Considering 5 × 10E6 CD34+ cells/kg a typical clinical transplant dose, a recipient would have received 16.6 ± 2.9 × 10E3 (range: 6–34.6 × 10E3) T cells/kg, a dose well within the specification (50 × 10E3/kg) of the licensed allogeneic CD34-selected stem cell product. Additional product properties are shown in Table I. Figure 2 demonstrates selected panels from a representative flow cytometric analysis of apheresis product, non-target (negative fraction) and target populations.

To establish at the same time optimal storage conditions and shelf life, as well as freezability, aliquots of product were stored at 4°C for 24–72 h in 0.5–5% HSA and subsequently analyzed for viable CD34+ cell recovery using the SCE kit. Surprisingly, HSA concentrations of 0.5% were associated with the highest recovery, 81 ± 9% and 78 ± 8% after 48 and 72 h. Detailed stability data are shown in Figure 3. Aliquots of CD34-selected cells stored in 5% HSA were also supplemented 24 h after the apheresis end (to mimic a worst-case clinical scenario for cryopreservation) with cryo-medium to a final concentration of 50% saline with 5% w/v HSA, 40% X-Vivo10 and 10% dimethyl sulfoxide and frozen in controlled-rate freezers. After at least 24 h in storage at −145°C, an aliquot was thawed and subjected to viable CD34+ cell enumeration. Of the viable CD34+ cells contained in the aliquot collected immediately pre-freeze (71 ± 7% of that immediately after the end of the process), 82 ± 14% were recovered post-thaw. Thus, stability of Prodigy-processed CD34+ cells for the specified shelf life as well as suitability for freeze-thawing was documented.

Discussion

This formal validation exercise of the new fully automatic CD34+ cell selection process with nine full-scale mobilized apheresis products on the CliniMACS Prodigy device was performed under routine clinical conditions, even though none of the cell products were slated for clinical use. Results therefore can be immediately translated into clinical routine. Indeed these data concurrently served as validation runs, which the manufacturer submitted for approval of the CE mark from the national authority for the CD34 process on Prodigy. The "process" consists of the reagents that are the same as those used for the CD34 process on the CliniMACS, the Prodigy consumable (tubing set) and the specific CD34 selection software. As reported, the entire process proceeded flawlessly without unscheduled operator intervention.

Analysis of the target population reveals a depletion factor (ratio of T cells after and before selection) that is slightly inferior, albeit not statistically, to that achieved with concurrently performed clinical CliniMACS Plus CD34 processes. However, as a result of how donors for the validation study were selected, the T-cell frequency was much lower (by about half) in the Prodigy cohort. This variable

Table I. Properties of apheresis products and CD34-selected cell products generated there from by CliniMACS Prodigy (experimental) or concurrent CliniMACS Plus (clinical) (mean±SEM).

	CliniMACS Prodigy	CliniMACS Plus
Apheresis product		
WBC (total)	4.75 ± 0.5 × 10E10	5.35 ± 0.8 × 10E10
CD34+	4.78 ± 0.6 × 10E8	4.12 ± 0.5 × 10E8
CD3+	9.58 ± 0.8 × 10E9	2.04 ± 0.3 × 10E10
CD4+	6.01 ± 0.5 × 10E9	—
CD8+	3.13 ± 0.2 × 10E9	—
CD14+	1.19 ± 0.08 × 10E10	—
CD20+	2.08 ± 0.3 × 10E9	—
CD56+	1.59 ± 0.3 × 10E9	—
CD34+ frequency	1.21 ± 0.26%	0.63 ± 0.04%
CD34+ viability	99.85 ± 0.06%	99.96 ± 0.04%
Post-selection		
WBC (total)	3.54 ± 0.5 × 10E8	3.06 ± 0.3 × 10E8
CD34+	3.42 ± 0.5 × 10E8	2.93 ± 0.3 × 10E8
CD3+	9.44 ± 1.3 × 10E5	3.18 ± 1.7 × 10E5
CD4+	5.42 ± 0.8 × 10E5	—
CD8+	3.35 ± 0.5 × 10E5	—
CD14+	1.22 ± 0.4 × 10E6	—
CD20+	3.64 ± 0.5 × 10E6	—
CD56+	4.04 ± 1.2 × 10E5	—
CD34+ frequency	93.87 ± 0.84%	95.96 ± 1.23%
CD34+ viability	99.85 ± 0.05%	99.78 ± 0.08%
CD34+ recovery	73.6 ± 13.2%	72.4 ± 2.8%
CD3+/5 × 10E6 CD34+	16.6 ± 2.9 × 10E3	9.01 ± 5.8 × 10E3
CD3+ depletion	9.7 × 10E-5 ± 7.7 × 10E-6	4.2 × 10E-5 ± 3.1 × 10E-5
CD20+/5 × 10E5 CD34+	7.79 ± 2.2 × 10E4	—

alone affects the "depletion factor" for T cells, although independent of the selection technology. Clearly, the suggested modest advantage of CliniMACS Plus with respect to the depletion factor (9.7 × 10E-5 ± 7.7 × 10E-6 for Prodigy versus 4.2 × 10E-5 ± 3.1 × 10E-5 for CliniMACS Plus, $P = 0.14$) is only partly due to this imbalance between starting materials. The hypothetical "per kilogram" T-cell dose that a recipient would have received, assuming a stem cell dose of 5 × 10E6/kg BW, also trended toward higher values with Prodigy (16.6 ± 2.9 × 10E3) than with CliniMACS (9.0 ± 5.8 × 10E3, $P = 0.3$). Because the number of T cells in the product is higher for Prodigy, the average recipient would receive more T cells or, if a more stringent limit for T cells is assumed, − fewer CD34+ cells/kg than with a CliniMACS Plus product. Why final product variability with respect to recovery was greater with the automatic method than with the semi-automatic predecessor technology is not clear; it is apparent that the operator has no influence over this outcome. The markedly higher neutrophil content of the starting apheresis material might be partly responsible because neutrophils are known to negatively affect both purity and recovery of immunomagnetic methods. Alternatively, platelet contamination has been reported to affect purity and recovery; data generated during the development of the CD34 selection protocol on Prodigy confirm, however, that the three successive automatic platelet washes on Prodigy remove 50−70% of platelets, which is similar to the efficiency of the CliniMACS manual platelet washes.

As is apparent from Figure 1, Prodigy only minimally reduces process time over CliniMACS Plus, but the hands-on operator time is reduced by almost 2 h. Because the Prodigy process could run unsupervised or supervised remotely overnight, a possible advantage would be avoidance of unfavorable working hours while recovering cells of maximal freshness and, hence, functional quality, given that experience shows approximately linear cell loss over time, starting from the moment that the cells are collected from the donor [21]. The process of CD34 selection is maximally simplified by Prodigy.

Conclusions

This is the first report on the CD34 selection process on CliniMACS Prodigy. As we have shown, CliniMACS Prodigy is unconditionally suitable to perform the CD34 selection process; all nine validation products met the pre-defined specification of the licensed cell product G-CSF mobilized allogeneic HPCs, CD34 selected, German Red Cross Blood Service Baden-Württemberg-Hessen. Recovery of

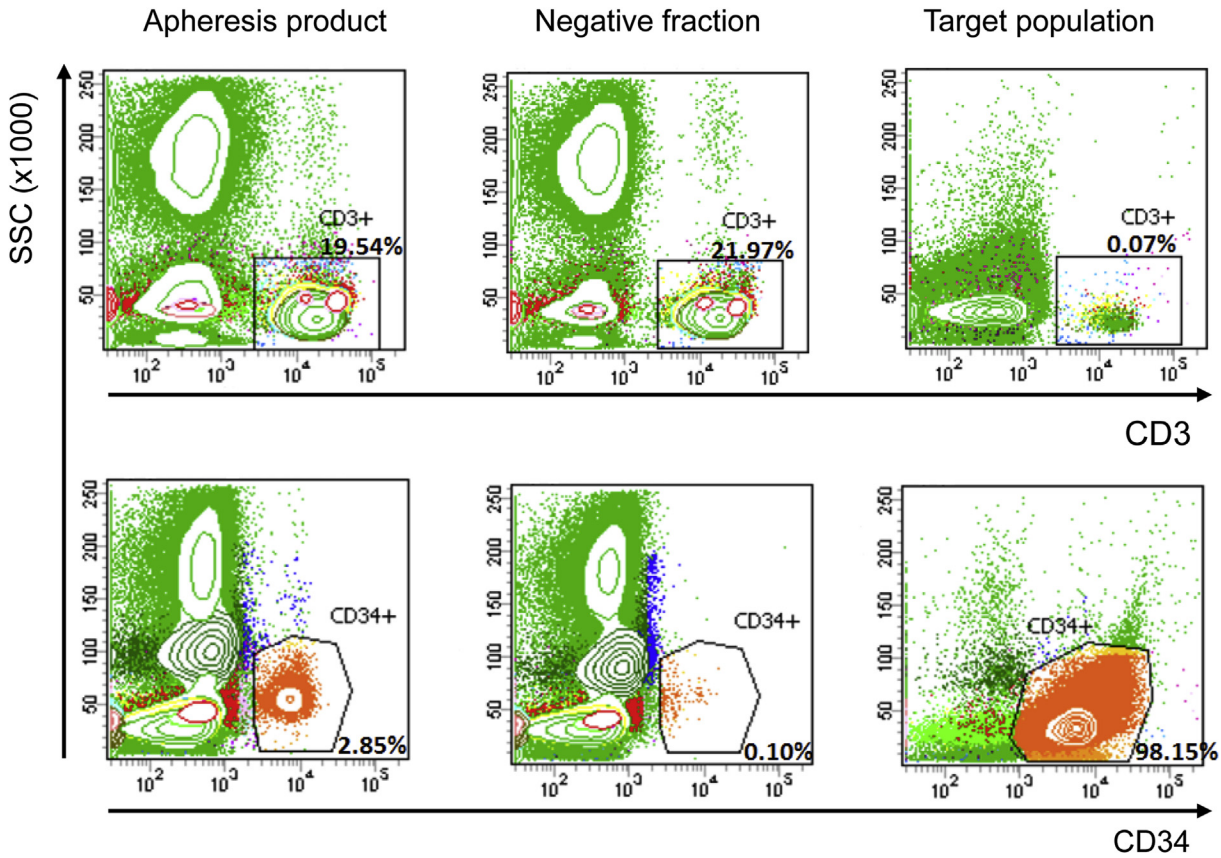

Figure 2. Quality analysis of Prodigy-generated CD34 selected products: partial depiction of residual T-cell enumeration by flow cytometry. The representative example shows in the apheresis product a sevenfold excess of T cells over CD34+ cells; the final product contains 1400 CD34+ cells for every T cell, yielding a T-cell depletion factor relative to the target cells of 10E-4 (4 log).

target cells was equal to that reported for the semi-automatic method. Final product content of potentially allo-reactive T cells was modestly higher, albeit not statistically; preliminary studies indicate that it could be further improved by more copious flushing of the column and the complex tubing system. Prodigy reduces operator time by approximately half. Once all other cell products currently generated on CliniMACS Plus can be similarly produced on Prodigy, regulatory approval processes are completed and an open software permitting cell therapy laboratories "programming" their own cell processing processes becomes available, CliniMACS Prodigy can replace CliniMACS Plus in cell processing facilities. Although in principle it is simple enough for any transplant unit to use, the quality control (residual T-cell quantification) is demanding and will limit the technology to a finite number of centers of excellence. Because, as we have shown, Prodigy products are quite stable when refrigerated, worldwide shipping from a small number of sites would be conceivable.

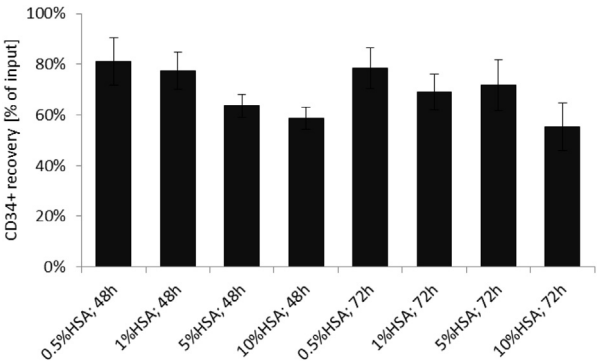

Figure 3. Stability of Prodigy-generated CD34 products in different media: stability data for CD34+ cells in 0.5–10% HSA in saline stored at 4–8°C for 48 h or 72 h after the end of the apheresis are shown. Data present the concentration of viable CD34+ cells recovered at the indicated time points as percent of the concentration measured immediately post-manufacturing. Stability is excellent for the entire observation time; it is best preserved in HSA 0.5% in saline.

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gifted by Miltenyi Biotec. No outside funding was used for these studies.

MB, CH, EW, NS and CP performed separations. GS, EW and DK performed cytometric analyses. GS and HB analyzed the data. JS, KR, VH and ME developed the technology described here and were involved in study conception and data analysis. HB conceived of the studies and wrote the manuscript. ES and HB share the overall responsibility.

Disclosure of interest: HB has received honoraria from Miltenyi, manufacturer of the CliniMACS Plus and CliniMACS Prodigy systems. JS, KR, VH and ME are employees of Miltenyi and were involved in the development of the protocol tested here. The other authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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