International Society for Cellular Therapy ISCT

Cytotherapy (2006) Vol. 8, No. 5, 465–472

Taylor & Francis
Taylor & Francis Group

Determination of residual T- and B-cell content after immunomagnetic depletion: proposal for flow cytometric analysis and results from 103 separations

M Schumm[1], R Handgretinger[1], M Pfeiffer[1], T Feuchtinger[1], S Kuci[1], C Faul[2], W Vogel[2], W Bethge[2] and P Lang[1]

[1]Children's University Hospital Tübingen and [2]Department of Hematology and Oncology, University Hospital of Internal Medicine, University Hospital Tübingen, Tübingen, Germany

Background

T- and B-cell depletion of apheresis products is an attractive alternative to standard stem cell enrichment in haplo-identical transplantation. Thorough T- and B-cell depletion is necessary for prevention of acute GvHD and T-cell depletion-associated lymphoproliferative disorders. However, the large number of non-T and -B cells in the graft requires special protocols for the determination of extremely low frequencies of residual T cells.

Methods

Apheresis products from healthy donors were T- and B-cell depleted by the CliniMACS™ system using CD3 and CD19 Ab reagents and the LS tubing set. The recovery of cells and degree of depletion were determined. A four-color multigating strategy was used for enumeration of residual T and B cells

Results

One-hundred and three separations were performed, with a mean cell recovery of 38 ± 12%, CD34 recovery of 61 ± 16% and CD56 recovery of 63 ± 33%. T and B cells were depleted by log 4.15 ± 0.46 and log 3.64 ± 0.63, respectively. Four-color multigating flow cytometry allowed the detection of single T cells.

Discussion

Combined T- and B-cell depletion is a feasible method for obtaining stem cell grafts with acceptable stem cell recovery, profound T- and B-cell depletion and a very high amount of NK cells and monocytes. However, analysis of residual T cells is challenging and requires special protocols.

Keywords

allogeneic transplantation, B cell, CliniMACS, depletion, flow cytometry, T cell.

Introduction

T-cell depletion is a pre-requisite for stem cell transplantation from HLA-mismatched donors to prevent severe GvHD. In most centers T-cell depletion is achieved passively by enrichment of $CD34^+$ or $CD133^+$ stem cells [1]. The technique became standard in many centers because it allows fast and effective depletion of T cells with an acceptable recovery of stem cells. Haplo-identical transplantation has been made feasible by the use of high numbers of stem cells (the megadose concept) together with an intensive conditioning regimen [2,3]. However, a megadose of stem cells is sometimes difficult to achieve, particularly for adult patients. The profound T-cell depletion results in delayed immune reconstitution leading to a high rate of infectious complications [4].

To improve immune reconstitution and enhance engraftment, new strategies of graft manipulation are necessary. One strategy could be selective CD3 and CD19 depletion, resulting in grafts not only containing $CD34^+$ stem cells but also $CD34^-$ progenitors, NK-, dendritic- and graft-facilitating cells. Direct depletion of T cells from BM has been used initially in BM transplanta-

Correspondence to: **Michael Schumm**, Children's University Hospital, Hoppe-Seyler-Str. 1, 72076 Tübingen, Germany. E-mail michael.schumm@med.uni-tuebingen.de.

DOI: 10.1080/14653240600932787

tion but the number of T cells in an apheresis product is tenfold higher than in BM and the techniques used are less effective and in most cases not GMP compliant. Initial *in vitro* data showed that the CliniMACS™ technique was also suitable for the depletion of apheresis products, with high efficiency and acceptable stem cell recoveries [5]. We therefore used the CliniMACS method with directly labeled CD3 and CD19 magnetic microbeads for depletion of apheresis products in haplo-identical transplantation. B-cell depletion was performed to avoid lymphoproliferative complications after transplantation. The first experiments showed that the procedure was much more demanding than enrichment and, in particular, the analysis of residual T cells required special protocols that were able to detect single cells in up to 1×10^5 cells. We therefore developed a four-color multigating protocol including positive and negative markers of T cells and viability staining of the cells. Up to now 103 separations have been performed successfully and enabled transplantations in both pediatric and adults patients, with promising results in terms of engraftment, the ability to reduce the conditioning regimen and enhanced immune reconstitution that has resulted in reduced transplant related mortality [4].

Methods

Stem cell mobilization and collection

Apheresis products were collected from 56 healthy donors for allogeneic haplo-identical transplantation. Stem cells were collected after mobilization with 10 μg/kg G-CSF injected s.c. daily for 5 days, and leukapheresis was performed on days 5 and 6 using a Cobe Spectra (Gambro, Lakewood, CO, USA). Apheresis products were stored overnight at 4°C and separated the next day. Depletions of apheresis products were performed in compliance with the German drug law with informed consent of the donors.

Depletion with the CliniMACS device

Depletion was performed according to the manufacturer's instructions, with minor modifications (Miltenyi Biotec, Bergisch-Gladbach, Germany). The amount of CliniMACS reagent was used as recommended: 1 vial of CD3 and CD19 CliniMACS reagent for 4×10^{10} MNC with a maximum of 15×10^9 $CD3^+$ cells and 5×10^9 $CD19^+$ cells. Apheresis products were transferred into 175-mL centrifuge tubes (Nalgen Nunc, Wiesbaden, Germany). The tubes were filled with DPBS (Biochrom, Berlin, Germany) supplemented with 0.8% human albumin and 5 mmol/L K-EDTA (Central Pharmacy, University of Tübingen, Tübingen, Germany). Up to 2×10^{10} nucleated cells/tube were centrifuged for 15 min at $400 \times g$. The supernatant was discarded and cells were resuspended with washing buffer to a final volume of 45 mL; 3.85 mL of CD3 and CD19 reagent were added per tube, and cells were incubated at room temperature. Tubes were then filled with washing buffer, centrifuged again for 15 min at $400 \times g$ and the supernatant discarded. The volume was adjusted to a defined value (according to the manufacturer's recommendations) and cells were transferred, after careful resuspension, to a 400-mL transfer bag (Baxter, Unterschleissheim,
Germany).

The CliniMACS device was started with program depletion 2.1 and the values of the sample (percentage of target cells, cell concentration and volume) were entered into the program as indicated. After automatic processing, cell samples were taken for subsequent analysis.

Flow cytometric analysis

Apheresis product and depleted cells were analyzed by flow cytometry; 50 000 events were acquired for all Ag except for CD3 and CD19/20 in the depleted fraction. Here, 1×10^6 events were acquired to address the low frequency of T and B cells after depletion. In addition, an analysis of T cells was performed with two samples. Cells were analyzed for CD34, CD133, CD14, CD16/56, CD19 and CD3. Ab combinations for analysis are given in Table 1.

The gating was as follows. Gate 1 was set to include all cells to enable calculation of absolute cell numbers from the value of the hematology analyzer. Gate 2 included lymphocytes. Gate 3 excluded propidium iodide-positive cells and cells expressing CD14 and CD33. Gate 4 was set using the cell sample from the negative fraction spiked with a small amount (about 3%) of the positive fraction. The gate was set to include all $CD3^+$ cells. Clustering of cells as $CD4/8^+$ events improved the appearance on the plot as a distinct population (Figure 1)

Results

Performance of system

Separation runs were performed by the device without any complications. As calculated by the software, several loading stages could be observed where positive cells