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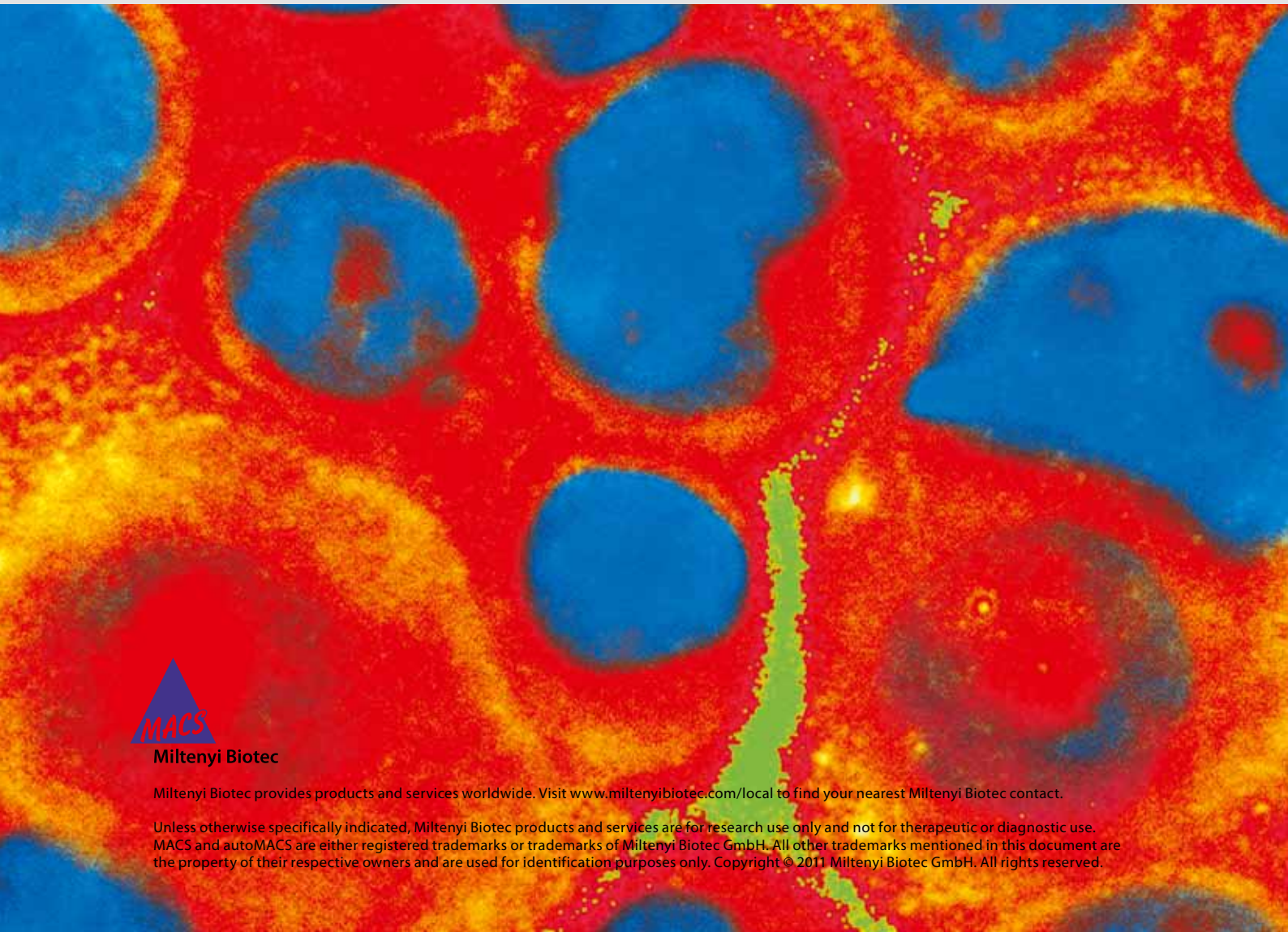

Enrichment of cell subpopulations by MACS® Technology for PCR-based chimerism analysis

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Introduction

Allogeneic stem cell transplantation (SCT) has become an established mode for treatment of different hematological malignancies. Monitoring engraftment is a key part of post-transplant treatment. In the early post-transplant period after allogeneic SCT, co-existence of host and donor cells can develop, especially in case of a reduced intensity conditioning (RIC) regimen. This status is referred to as mixed chimerism, whereas complete donor chimerism denotes the situation where all cell lineages are reconstituted by donor-derived cells. Thus, chimerism analysis provides crucial information on the progress of donor cell engraftment or failure following allogeneic SCT[1–7]. An increase of mixed hematopoietic chimerism can lead to a reduction of the graft-versus-leukemia effect, thus leading to proliferation of malignant host cells that were not eliminated during the conditioning procedure. Assessment of the chimeric state of certain cell subpopulations may even be predictive for early graft rejection, graft-versus-host disease (GvHD), or imminent relapse [4,8–15]. To this end it is crucial to isolate particular cell populations to a degree that allows reliable chimerism analysis. In this study we show that MACS Technology using Whole Blood MicroBeads and the autoMACS® Separator from Miltenyi Biotec allows the automated isolation of cell subpopulations from post-transplant peripheral blood. The procedure is fast and provides excellent purity and recovery for reliable routine lineage-specific chimerism analysis if the absolute cell count of the purified sample is larger than 400 per µL.

Materials and methods

Samples

137 peripheral blood (PB) samples were collected from 15 pediatric patients who received allogeneic stem cell transplants. Transplants were depleted of $CD3^+$ and $CD19^+$ cells. Thirteen patients received RIC and two patients received myeloablative conditioning prior to SCT. Samples were collected and analyzed weekly.

Magnetic enrichment of PB cell subpopulations

Lineage-specific cell enrichment was performed by using CD3, CD14, CD15, CD19, and CD56 Whole Blood MicroBeads (Miltenyi Biotec), yielding T cells, monocytes, granulocytes, B cells, and natural killer (NK) cells, respectively.[16]

Between 500 µL and 1 mL of PB was used for the enrichment of each subpopulation. Cells were magnetically labeled by adding 50 µL Whole Blood MicroBeads per 1 mL of PB. Cells were incubated at 4–8 °C for 15 min and washed subsequently by adding 5 mL of autoMACS® Running Buffer (Miltenyi Biotec) per 1 mL of PB. Cells were centrifuged and resuspended in one volume of buffer. Cell separation with the autoMACS Separator was carried out according to the user manual applying the program 'posseld2'. The positive fraction containing the desired cell type was divided into two samples. One of them was used for flow cytometric analysis to evaluate purity and recovery of the cell subpopulations, the other one allowed DNA isolation for subsequent chimerism analysis.

Chimerism assays

Hematopoietic chimerism was assessed either by the commonly used PCR-based amplification of short tandem repeats (STR-PCR)[1,17] or by real-time quantitative PCR based on sequence polymorphisms (SP-qPCR)[18].

Results and discussion

Purity and recovery of cell subpopulations enriched by MACS Technology

The results of cell separation are summarized in table 1. $CD\ 14^+$ monocytes showed a median purity of 96.4% (n=106) with a median absolute cell count (ACC) of 401 cells/µL.

Purities greater than 90% were achieved in 81% of the samples.

CD15⁺ neutrophils showed a median purity of 98.7% (n=98). The median ACC amounted to 2,781 cells/µL. Purities greater than 90% were achieved in 95% of the samples.

CD3⁺ T cells were enriched to a median purity of 90.1% (n=115) with a median ACC of 393 cells/µL. The purity depended on the ACC. In samples with an ACC >400 cells/µL a 90% purity was achieved in 86% of the samples (n=57).

CD19⁺ B cells had a median purity of 40.9% and a median ACC of 7 cells/µL. Purity was again dependent on the ACC. When the ACC was >400 cells/µL, a 90% purity was achieved in 86% of the samples (n=7).

It is noteworthy that adequate purities of CD3⁺ or CD19⁺ cells were only achieved if the ACC was higher than 400 per µL (fig. 1). It should be considered that CD3⁺ and CD19⁺ cells were depleted from the transplant and, therefore, the number of these cells might have remained very low early after SCT. This indicates that limited cell numbers of a specific population might hamper lineage-specific chimerism analysis and points to the importance of determining cell counts of the samples.

CD56⁺ NK cells showed a median purity of 78.9% and an ACC of 201 cells/µL. The purity showed the tendency to increase with the ACC. However, a threshold ACC, above which a purity >90% was commonly obtained, could not be determined for the CD56⁺ isolates.

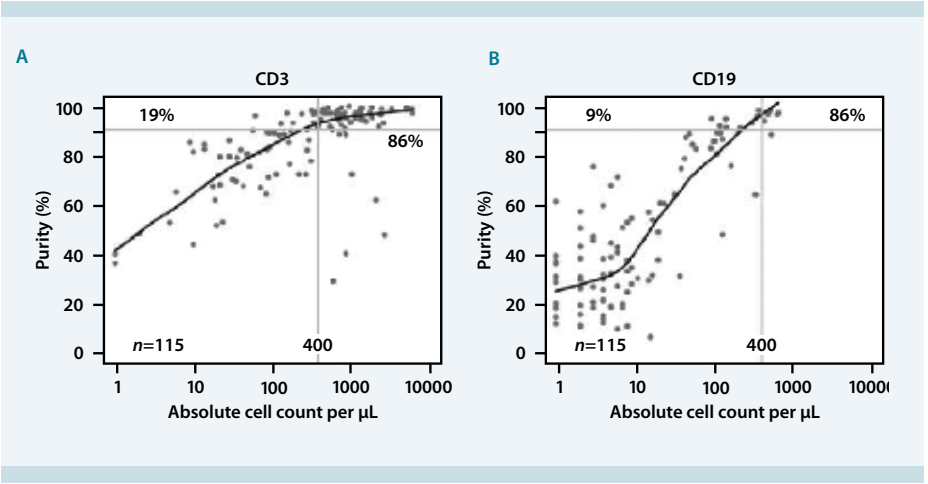

Figure 1 **Purity of subpopulations.** Dots indicate the raw values measured in each individual patient's sample. Smoothened curves indicate a trend that was derived from locally weighted regression with a smoother span of f=2/3 performed with the LOWESS function.

CD56⁺, CD14⁺, and CD19⁺ cells showed recoveries of >90% in 93%, 81%, and 80% of the samples, respectively. More than 70% recoveries were observed for CD3⁺ and CD15⁺ cells in 89% and 63% of the samples (table 1). The apparent lower recovery of the CD15⁺ cells may be attributed to the high initial cell numbers of this subset.

DNA was isolated subsequently to the enrichment of CD3⁺, CD14⁺, CD15⁺, CD19⁺ and CD56⁺ cells. The DNA concentration amounted to at least 0.5 ng/µL in the vast majority of samples, which was a prerequisite for dependable chimerism analysis by qPCR.

Feasibility of chimerism analysis in cell subpopulations

In general, feasibility of both STR-PCR- and SP-qPCR-based chimerism analyses of cell subpopulations isolated by MACS Technology was excellent. STR-PCR-based chimerism analysis was possible in the vast majority of the cell subpopulations, ranging from 98.2% (CD3⁺ cells) to 90.3% (CD19⁺ cells). Similarly, SP-qPCR-based analysis was possible in 100% (CD3⁺, CD14⁺ cells) to 95.2% (CD19⁺ cells) of the processed samples (table 1).

Cell population	Number of samples	Recovery[a] after cell separation ≤90 %	90–70 %	<70 %	Purity after cell separation; median in % (range)	Purity >90% after cell separation in %	ACC after cell separation; median in cells per µL (range)	STR-PCR chimerism analysis feasible in %	Samples with DNA concentration ≥0.5 ng/µL n (%)	SP-qPCR[b] chimerism analysis feasible in %
Whole PB	137	–	–	–	–	–	–	100.0	136 (99.3)	100.0
CD3⁺	135	62	27	11	90.1 (29.6–99.6)	86[c]	393 (1–6.268)	98.2	110 (81.5)	100.0
CD19⁺	137	80	9	11	40.9 (7.5–97.9)	86[c]	7 (1–647)	90.3	62 (45.3)	95.2
CD14⁺	119	81	11	8	96.4 (64.7–99.6)	81	401 (26–1.313)	95.7	117 (98.3)	100.0
CD15⁺	109	37	26	37	98.7 (73.7–99.9)	95	2.781 (308–18.170)	96.0	101 (92.7)	98.0
CD56⁺	132	93	5	2	78.9 (24.6–95.8)	14	201 (2–2.016)	92.7	123 (93.2)	97.6

Table 1 **Cell specimens—recovery, purity and feasibility of chimerism analysis.** ACC, absolute cell count; PB, peripheral blood; SP, sequence polymorphism; qPCR, quantitative real-time PCR. [a] Ratio of ACC after magnetic isolation and ACC in PB. [b] In samples with a DNA concentration ≥0.5 ng/mL. [c] If ACC was >400/mL.

Example of how lineage-specific chimerism may guide early therapeutic intervention after SCT

Lineage-specific chimerism analysis was performed using samples from a single individual who received SCT. The transplant was depleted of $CD3^+$ and $CD19^+$ cells. Analysis indicated that autologous signals in $CD3^+$ cells increased on day 33 and in PB on day 40. The autologous signal increased to 80% in the $CD3^+$ population, whereas in PB the signal increase was less prominent and amounted to only 10%. This indicates that chimerism analysis of distinct cell subpopulations may yield more clear-cut results than analysis of non-separated PB. Rapid tapering of the immunosuppressive drug mycophenolate mofetil (MMF) was initiated on day 56, which, however, did not reduce autologous signals. By contrast, donor lymphocyte infusions on day 142 and day 182 reconstituted complete donor chimerism in $CD3^+$ cells and PB, which persisted until the latest follow-up sample on day 820 (fig. 2).

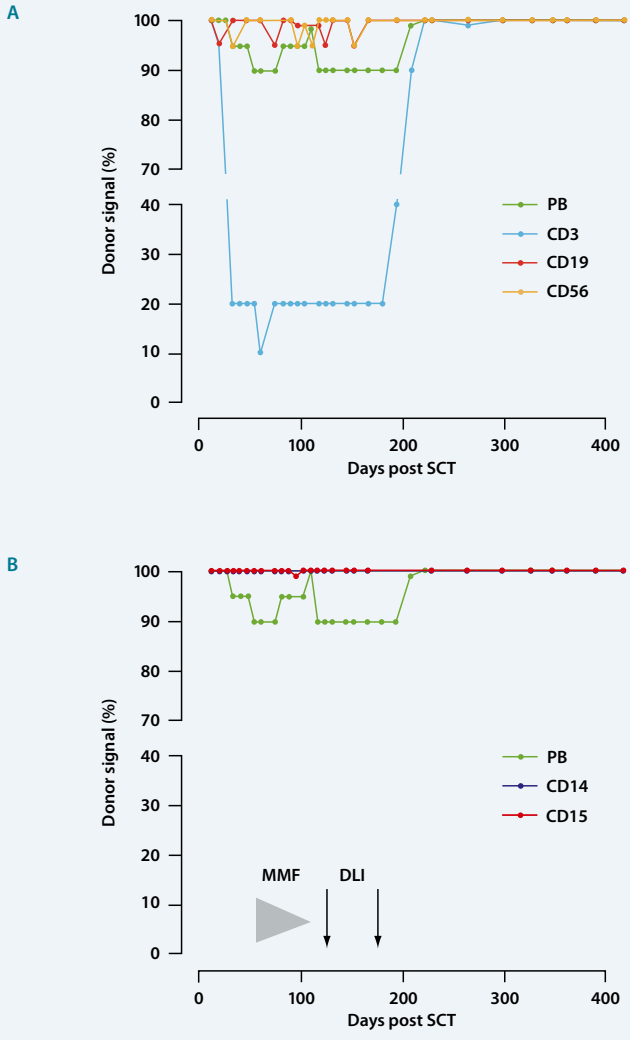

Figure 2 **Lineage-specific chimerism analysis in peripheral blood, mononuclear (upper graph) and myeloid cells (lower graph) showing response to immunotherapy.** MMF, mycophenolate mofetil; DLI, donor lymphocyte infusion.

Conclusion

Enrichment of cells by MACS Technology using Whole Blood MicroBeads and the autoMACS Separator yielded cell populations at high purity and recovery allowing routine lineage-specific chimerism analysis. The procedure is faster, easier, and more cost-effective compared to flow sorting.

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MACS® Product	Order no.
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Whole Blood CD14 MicroBeads, human	130-090-879
Whole Blood CD15 MicroBeads, human	130-091-058
Whole Blood CD19 MicroBeads, human	130-090-880
Whole Blood CD56 MicroBeads, human	130-090-875