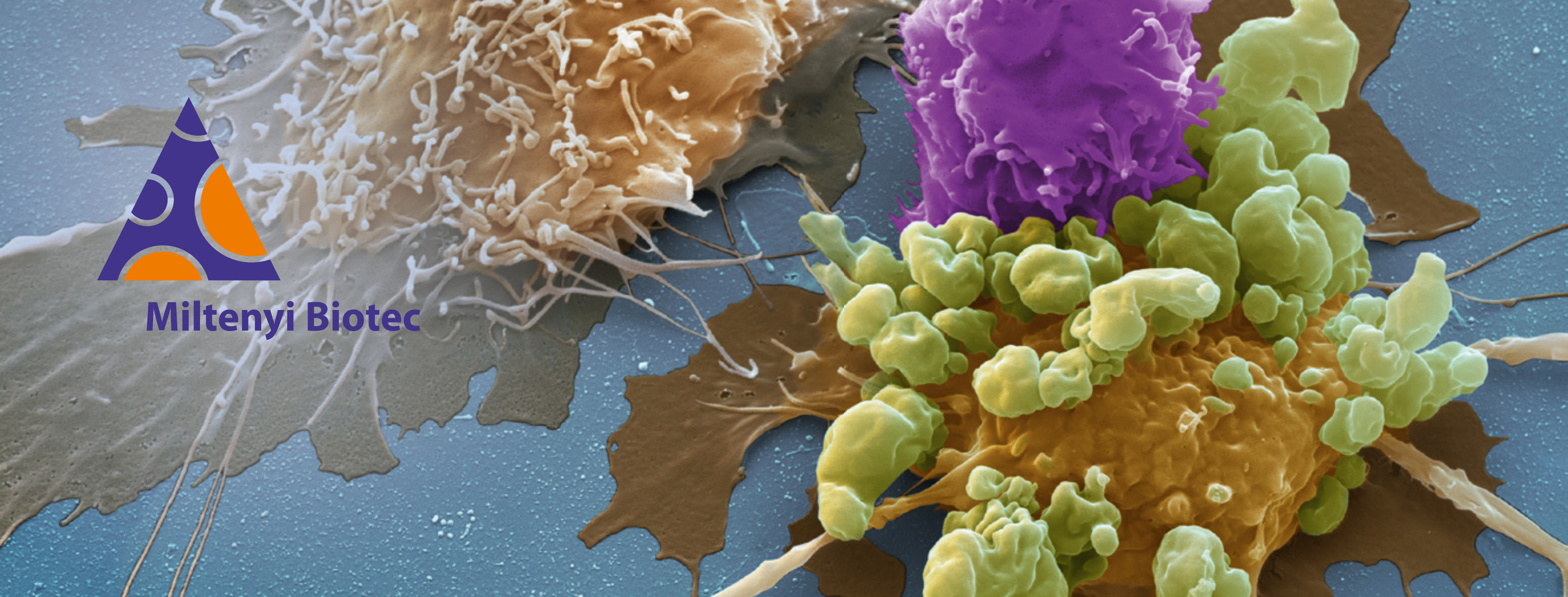

Fast and efficient T cell isolation without density gradient centrifugation for CAR T cell engineering

Background

In recent years, CAR T cell-mediated immunotherapy has shown high clinical relevance in the fight against cancer. However, increased research efforts to address challenges, such as poor efficacy and unwanted side effects, are required to further advance the field. At Miltenyi Biotec we have established optimized CAR T cell engineering workflows, combining cell isolation, culture, and analysis tailored for CAR T cell generation in different contexts. To generate fully functional CAR T cells, high quality T cell isolation is required. For successful and efficient magnetic cell isolation MACS® MicroBeads are the best choice. To enrich CD4⁺ and CD8⁺ T cells, either PBMCs can be generated first, followed by cell isolation with standard MicroBeads or T cells can be separated directly from blood products, such as whole blood, LRSC, buffy coat, or Leukopak® using StraightFrom® MicroBeads (fig. 1). When separating cells directly from blood, time-consuming and highly user-dependent density-gradient centrifugation can be omitted. Here we performed a side-by-side comparison of StraightFrom MicroBeads and standard MicroBeads for the generation of CAR T cells. After T cell isolation, the immune cell composition, T cell activation status, transduction efficiency, and the CAR T cell functionality were compared (see workflow overview in figure 1). By using the isolation of T cells directly from blood products the experimental procedure is accelerated and allows for automation on platforms, like the autoMACS® Pro Separator, the MultiMACS™ Cell 24 Separator Plus and the MultiMACS X, while maintaining the quality, functionality, and downstream compatibility of the isolated T cells.

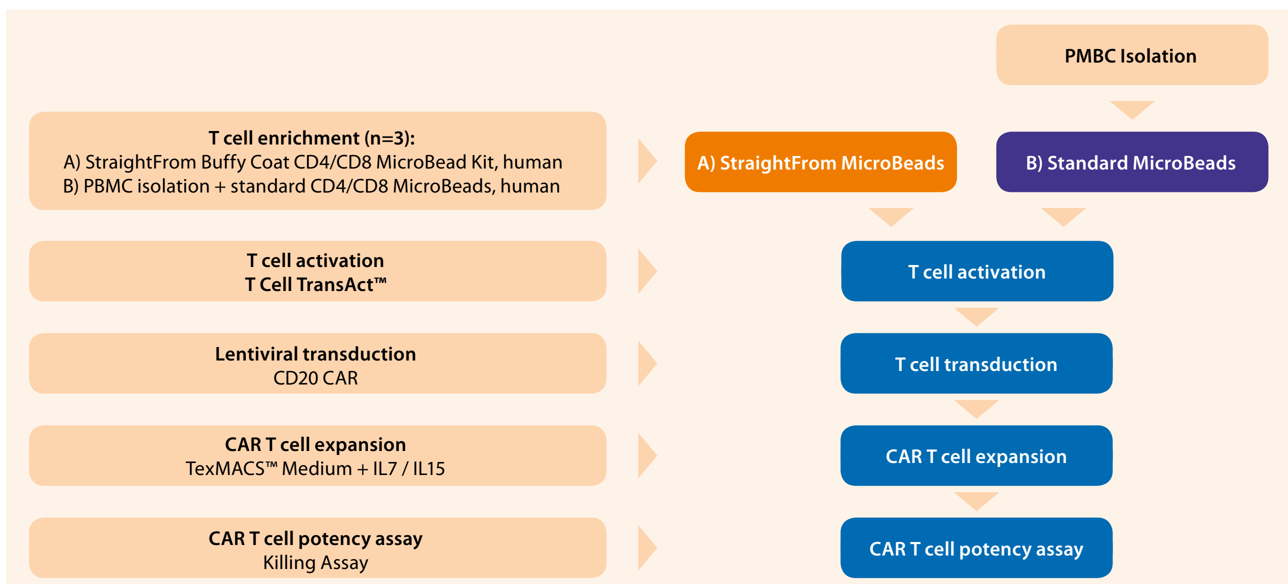

Figure 1: Experimental workflow overview.

Methods

For a side-by-side comparison of the downstream compatibility of standard MicroBeads and StraightFrom® MicroBeads, buffy coats of three independent healthy donors were used. CD4⁺ and CD8⁺ T cells were either isolated by direct separation using the StraightFrom Buffy Coat CD4/CD8 MicroBead Kit and the MultiMACS™ Cell24 Separator Plus (fig. 2) or by density-gradient centrifugation to isolate PBMCs followed by target cell separation using a 1:1 mixture of our standard CD4⁺ and CD8⁺ MicroBeads. Before and after magnetic T cell enrichment, the immune cell composition of each sample was analyzed by flow analysis using REAfinity™ Recombinant Antibody panels, the MACSQuant® Analyzer 10, and the CAR T Cell Express Mode package. Next, isolated CD4⁺ and CD8⁺ T cells were resuspended in TexMACS™ Medium and activated using the polyclonal T cell activation reagent T Cell TransAct™. This step is important for an optimal viral transduction and efficient T cell expansion. Expression of the T cell activation markers CD25 and CD69 was verified by flow analysis. One day after polyclonal stimulation, activated CD4⁺ and CD8⁺ T cells were transduced with a lentiviral vector encoding for a CD20 directed CAR or not transduced (mock control). Frequency of transduced, CD20 CAR⁺ T cells was measured directly after transduction (data not shown) and on day 13 after expansion in TexMACS Medium and MACS® Premium-Grade Cytokines IL-7 and IL-15. As a final step, antigen-specific target cell killing was assessed via a killing assay: CD20 CAR-transduced T cells were co-cultured with either the GFP⁺CD20⁺ JeKo-1 target cell line or a GFP⁺CD20⁻ JeKo-1 control cell line at effector to target cell (E:T) ratios of 5:1, 1:1, and 0.2:1 for 24 hours. Target cell killing was determined by analyzing the frequency of viable, green fluorescent protein (GFP) positive target cells at the respective E:T ratios.

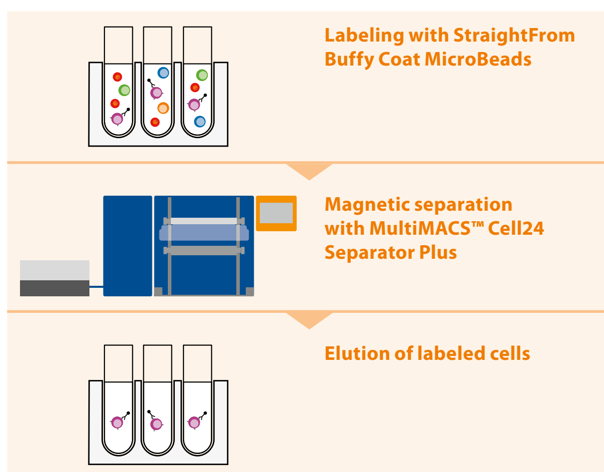

Figure 2: Automated cell isolation with the MultiMACS Cell24 Separator Plus.

Results

After enrichment using StraightFrom Buffy Coat CD4/CD8 MicroBeads, isolated CD4⁺ and CD8⁺ T cells exhibited a purity of 95%, 96%, and 94% for the three donors, whereas the percentage of other immune cell subsets (NK, NKT, monocytes, and B cells) amounted to 5%, 4%, and 6%, respectively (fig. 3). Comparing the T cell activation after polyclonal stimulation with T Cell TransAct™ of cells isolated with StraightFrom versus the standard MicroBeads, no significant difference in upregulation of CD25 and CD69 was observed (fig. 4). On day 13 after transduction and expansion, cells were analyzed for CD3 and the expression of CD20 CAR: 35.12% of the T cells isolated with StraightFrom MicroBeads and 39.1% of the T cells isolated with standard MicroBeads expressed the CD20 CAR, respectively, again exhibiting no significantly different results between the two separation approaches (fig. 5A, B).

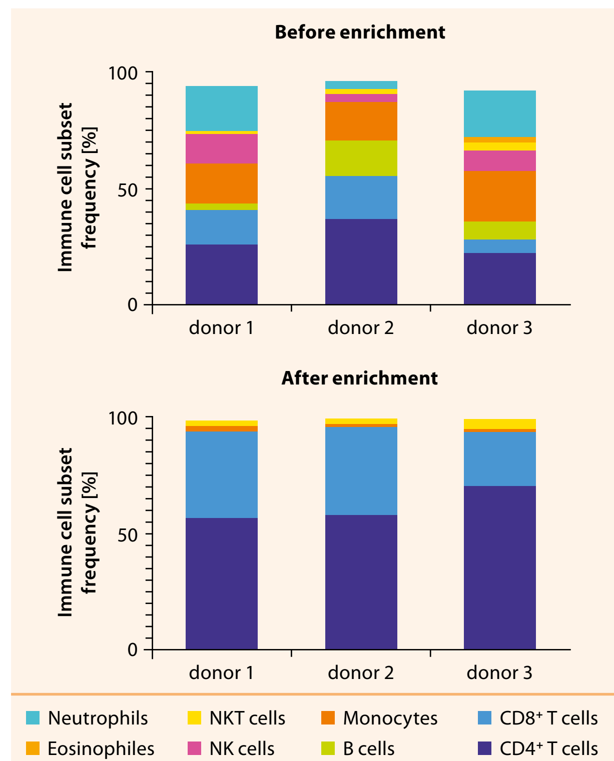

Figure 3: Immune cell composition of independent healthy donors (n=3) was determined before and after T cell enrichment with StraightFrom Buffy Coat CD4/CD8 MicroBeads using the MACSQuant Analyzer 10 and applying CAR T Cell Express Mode Package (Immune_Cell_Composition_human).

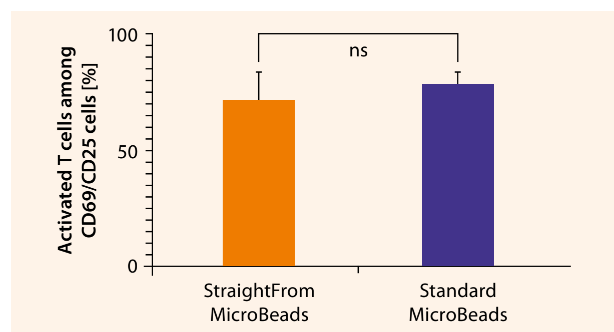

Figure 4: Frequency of activated T cells (24 hours after activation with T Cell TransAct) of independent healthy donors (n=3) was determined measuring the expression of the activation markers CD25 and CD69 among CD4⁺ and CD8⁺ cells using the MACSQuant Analyzer 10 and applying CAR T Cell Express Mode Package.

The CD20 CAR T cells that were generated using the different approaches were functionally tested in a killing assay. Flow cytometric analysis showed approximately 97% killing of target cells (GFP⁺CD20⁺ JeKo-1) at a 5-fold excess of CD20-directed CAR T cells (fig. 6) for both approaches (StraightFrom® MicroBeads and standard MicroBeads). When changing the ratio to 1:1 by decreasing the number of CAR T cells, a maximum of 50% of the target cells were killed. In contrast, the GFP⁺CD20⁻ JeKo-1 control cells were not killed by the CD20 CAR⁺ T cells, highlighting the specificity of the CAR T cells (data not shown).

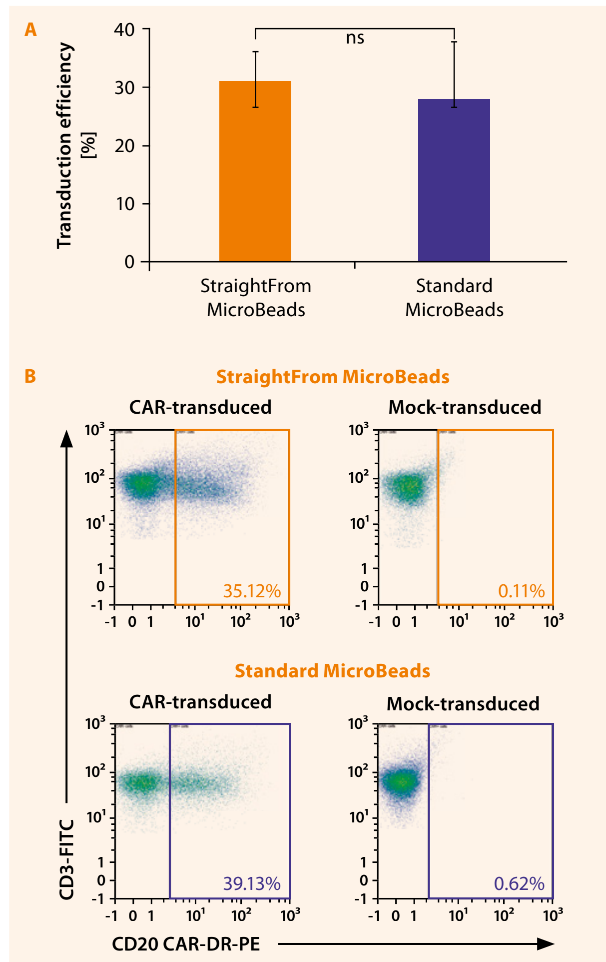

Figure 5: Lentiviral transduction efficiency on day 13 after transduction. A) Frequency of transduced T cells (13 days after transduction) from independent healthy donors (n=3) was determined measuring the measuring the expression of CD20 CAR on CD3⁺ T cells using the MACSQuant® Analyzer 10 and applying CAR T Cell Express Mode Package. B) Gating of acquired flow data exemplarily shown for donor 3 on day 13 after transduction.

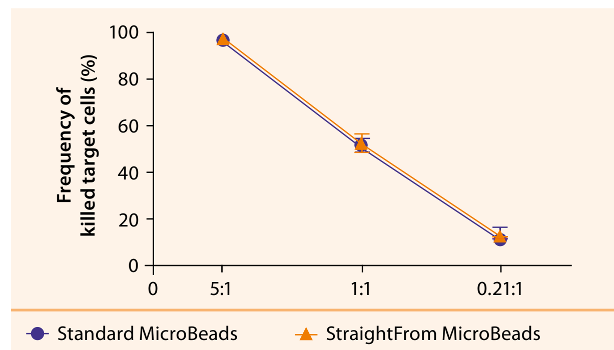

Figure 6: CAR T cell-mediated target cell killing. Frequency of killed target cells was determined by flow cytometry using the MACSQuant Analyzer 10. GFP⁺CD20⁺ JeKo-1 target cells were co-cultured with CD20-directed CAR T cells derived from independent healthy donors (n=3) at effector to target cell (E:T) ratios of 5:1, 1:1 and 0.2:1 for 24 hours.

Conclusions

A comparison of the standard MACS® MicroBeads and the StraightFrom® MicroBead Kits revealed that regardless of the isolation approach, magnetic cell separation using MACS Technology ensures preservation of T cell functionality enabling full downstream compatibility for CAR T cell generation. Using the innovative StraightFrom MicroBead solution to isolate CD4⁺ and CD8⁺ T cells directly from blood products allows the omission of density-gradient centrifugation for PBMC generation, thereby saving processing time and reducing user variability. In combination with options for automation, StraightFrom Technology is an ideal tool for streamlined and standardized experimental procedures. Experimental findings can then later be translated onto automated cell manufacturing platforms like the CliniMACS Prodigy®.

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Automated manufacturing of gene-engineered T cells under serum-free conditions

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Take a look at our detailed workflow protocol:
Engineering of CAR T cells for research use

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MACS® Product	Order no.
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CD8 MicroBeads, human	130-045-201
StraightFrom Buffy Coat CD4/CD8 MicroBead Kit, human	130-121-312
MultiMACS Cell24 Separator Plus	130-098-637
TexMACS Medium	130-097-196
Human IL-7, premium grade	130-095-361
Human IL-15, premium grade	130-095-762
T Cell TransAct, human	130-111-160
Vectofusin®-1	130-111-163
MACSQuant Analyzer 10	130-096-343
CAR T Cell Express Mode Package	160-002-376
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