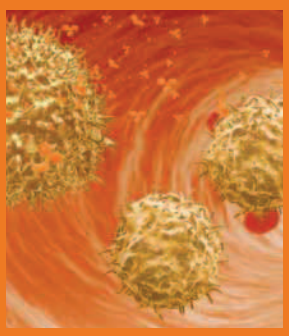
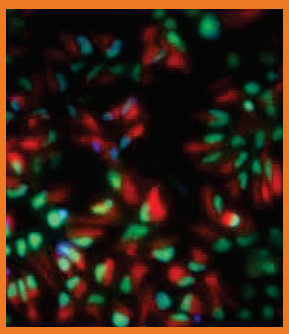
MACS & more
Vol 16 – 1/2014

Past and future innovation in cell therapy

25 years of Miltenyi Biotec

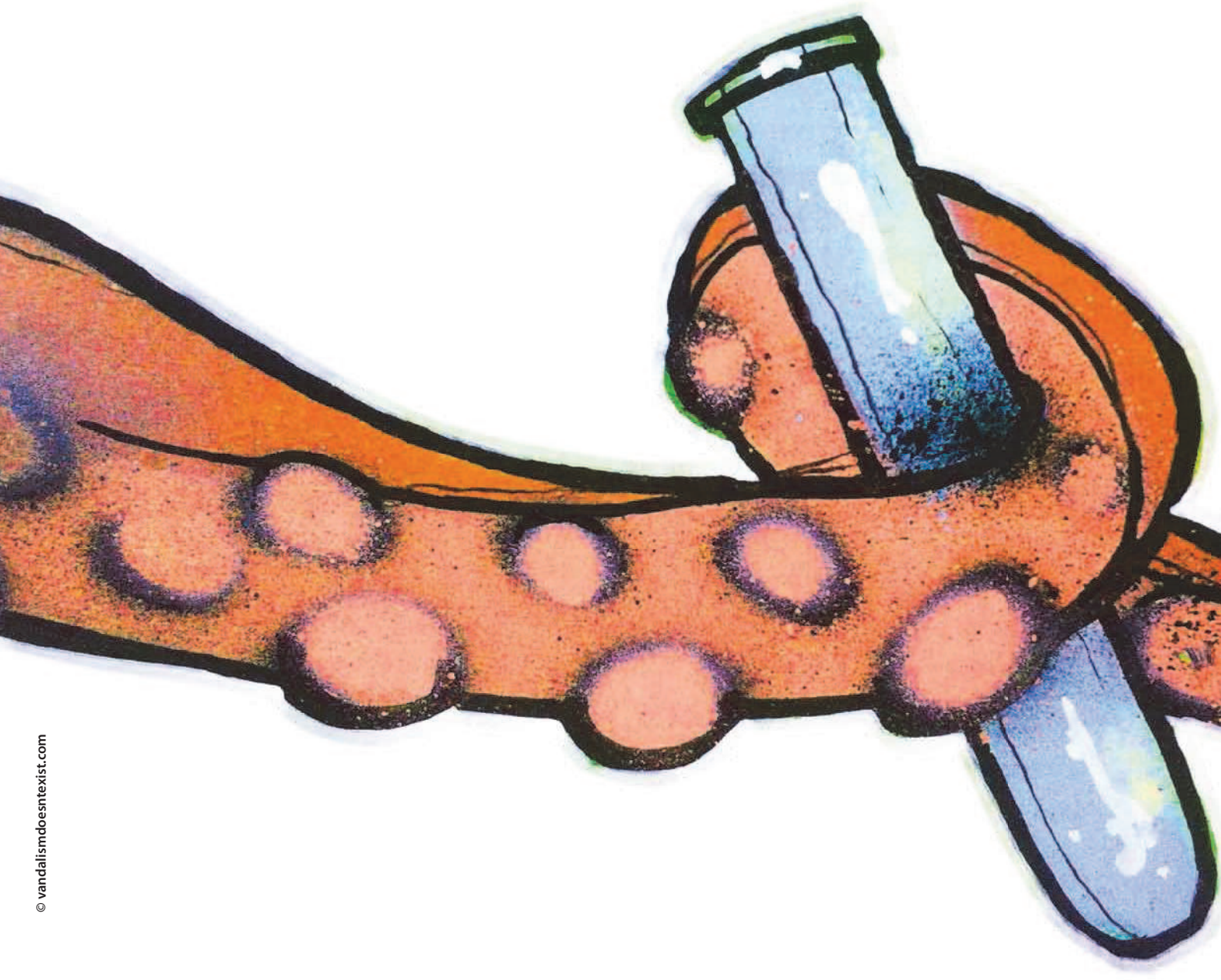


Tackling autoimmune diseases
Resetting the immune system is associated with long-term remission
▸ p. 24



Neural cells for tissue regeneration research
Differentiation from PSCs and subsequent isolation
▸ p. 29

Chimeric antigen receptor–expressing T cells
Holding great promise for cancer immunotherapy
▸ p. 32



Editorial 5

News

The way to integrated cell processing – 25 years of development and innovation at Miltenyi Biotec 6

A leap forward in cancer research 9

Reports

An alliance with Miltenyi Biotec in the fight for tolerance: A venture into no man's land 11
Niloufar Safinia, Giovanna Lombardi, and Robert Lechler

NK cell transplantation as consolidation therapy in children with acute myeloid leukemia – results from a pilot study 15
Wing Leung

Resetting the immune system in severe autoimmune diseases with immunoablation followed by autologous stem cell transplantation using enriched CD34$^+$ cells is associated with long-term remissions – a 15-year single-center experience 24
Tobias Alexander, Falk Hiepe, Renate Arnold, and Andreas Radbruch

Perspectives

Sorting for cell therapy 29
Xianmin Zeng and Mahendra Rao

Engineering CARs: How the idea of redirecting an immune response takes the front seat 32
Hinrich Abken

Science that stands out

Order your 25th anniversary autoMACS® Pro Separator. Select from 50 custom designs, hand-painted by local street artists. Additionally, you'll get an exclusive bonus package with your purchase that includes a year's supply of autoMACS Columns and a 2-year service plan.

▶ **miltenyibiotec.com/amp25**

Dear Researcher,

2014 is an exciting year for Miltenyi Biotec, as it marks the 25th anniversary of the company. This year of celebration coincides with major milestones in the company's history.

Earlier this year, the CliniMACS® CD34 Reagent System was approved by the FDA for GVHD prophylaxis in patients with acute myeloid leukemia (AML) in first complete remission undergoing allogeneic SCT from a matched related donor. This marks the culmination of many years of research and development, both at the bench and the bedside. Many people and organizations contributed to this achievement.

The development of the MACSQuant® Tyto™ will bring the world's fastest and most gentle flow sorter to the scientific community, opening up a new era for cell sorting. The instrument is designed around a microchip-based high-speed valve, and cell sorting occurs in a fully enclosed cartridge. We are confident that this technology will revolutionize cell sorting for many diagnostic and therapeutic applications in the future.

With the REAfinity™ Antibodies, Miltenyi Biotec introduced the next generation of flow reagents – recombinantly engineered clones that outperform all corresponding mouse and rat monoclonal antibodies. REAfinity Antibodies complement our existing portfolio of high-quality antibodies. They are available with our bright Vio® Dyes or traditional fluorochromes and represent the ideal choice for multicolor flow experiments.

These are exciting times for cellular therapy, as new strategies hold great promise for the treatment of diseases that are thus far incurable. We are happy that Lentigen Technology Inc. joined Miltenyi Biotec. With their long-standing expertise in the development of lentiviral technology, the Lentigen team will expand our existing portfolio of products for the development of innovative cell therapies involving CAR T cells, for example.

Back in 1989, it wasn't foreseeable that Miltenyi Biotec products would one day set benchmarks in so many disciplines, although we already had the vision of advancing biomedical research and cellular therapy.

The first tool offered by Miltenyi Biotec comprised superparamagnetic Biotin MicroBeads, different columns, and the first MACS® Separator, allowing for the versatile magnetic isolation of cells that are labeled with a biotin-conjugated antibody. This was the beginning of MACS Technology. Today, more than 7,000 products cover complete workflows – from sample preparation, via cell isolation, flow cytometry, cell sorting, and molecular analysis, through to cell culture. Our expertise spans research areas including immunology, stem cell biology, neuroscience, and cancer, and clinical research areas like immunotherapy and graft engineering, in addition to therapeutic apheresis. Over the years, hundreds of clinical trials towards cellular therapies, involving Miltenyi Biotec products, have been performed throughout the world.

Our vision would not have come to fruition without the numerous partners and customers, scientists and clinicians, across the world. I would like to take this opportunity to thank the many people who share our vision and who contribute to groundbreaking studies in biomedical research and cellular therapy – be it in basic research or the translation into clinical applications. We know that your collaboration and partnership is a huge token of trust, and you may rest assured that we will do our utmost to further strengthen your confidence in Miltenyi Biotec.

In this anniversary issue, we are thrilled to present a number of articles by distinguished scientists on cutting-edge research. Read about their results and perspectives on some of today's most promising approaches towards cellular therapies, involving regulatory T cells, NK cells, stem cells, neural cells, and CAR-expressing T cells.

I hope you enjoy reading the MACS&more anniversary edition and wish you all the best for your future research.

Kind regards,

Stefan Miltenyi

The way to integrated cell processing – 25 years of development and innovation at Miltenyi Biotec

For the development of cellular therapies it is essential to gain a profound insight into the specific function of individual cell types. To this end, it is often crucial to separate the target cells from heterogeneous cell mixtures. Cell isolation and further cell processing for clinical research has not always been as easy and effortless as it is now. Over the course of 25 years, Miltenyi Biotec revolutionized cell processing for both basic research and clinical application.

Opening up new options for cell therapy of leukemia: CliniMACS® CD34 Reagent System – the world's only FDA-approved device for GVHD prophylaxis

The potential of treating diseases by transplanting cells from a healthy donor into a patient was already recognized in the middle of the last century. Indeed, the first successful engraftment of bone marrow–derived cells was achieved in 1963 after allogeneic transplantation into a leukemia patient[1]. However, the patient subsequently suffered from chronic graft-versus-host disease (GVHD)[2] and died after 20 months, possibly due to GVHD-related complications.

Over the intervening years, intense basic and clinical research opened up a wide spectrum of potential cell therapies, including immunotherapy, tissue regeneration, and particularly hematopoietic stem cell transplantation (HSCT).

Since 1989 Miltenyi Biotec has played an essential role in the cell therapy community, providing sophisticated tools for the isolation of particular cell types, thus enabling the discovery of specific cell functions and the development of clinical applications. The options for HSCT in leukemia, for example, increased dramatically based on the CliniMACS® System. The device allows for the clinical-scale positive selection and enrichment of beneficial cells from grafts, such as $CD34^+$ cells repopulating the immune system after immune ablation. Likewise, the system enables the depletion of T and B cells that are known to contribute to GVHD. Over the years, more than 25,000 leukemia patients have been treated with cells manufactured with the CliniMACS System, and the approaches towards cellular therapy have been refined continuously.

In January of 2014, Miltenyi Biotec's constant quest for advancing biomedical research and cellular therapy bore fruit. The company received FDA approval for the CliniMACS CD34 Reagent System for GVHD prophylaxis in patients with acute myeloid leukemia (AML) in first complete remission undergoing allogeneic SCT from a matched related donor. The CliniMACS CD34 Reagent System as the sole means of GVHD prophylaxis decreases the risk of developing GVHD without negatively affecting relapse, engraftment, overall survival, or disease-free survival.[3,4] This device provides a new treatment option for patients who undergo a transplantation procedure.

Various reports at the ASH Meeting 2013[5–10] highlighted further promising clinical research in the field of graft engineering. Data involving selective depletion of $TCR\alpha/\beta^+$ cells from grafts to prevent GVHD were presented. The authors concluded that retaining $TCR\gamma/\delta^+$ T cells and NK cells in the graft may help achieve beneficial graft-versus-leukemia (GVL) effects, facilitate engraftment, and fight infections. This novel approach could provide even better treatment options in the future.

References

1. Mathé, G. *et al.* (1963) Br. Med. J. 2: 1633–1635.
2. Mathé, G. *et al.* (1965) Blood 25: 179–196.
3. Pasquini, M.C. *et al.* (2012) J. Clin. Oncol. 30: 3194–3201.
4. Devine, S.M. *et al.* (2011) Biol. Blood Marrow Transplant. 17: 1343–1351.
5. Prezioso, L. *et al.* (2013) Blood 122: 4609.
6. Dorn, C. *et al.* (2013) Blood 122: 2084.
7. Bertaina, A. *et al.* (2013) Blood 122: 157.
8. Airoldi, I. *et al.* (2013) Blood 122: 3245.
9. Te Boome, L. *et al.* (2013) Blood 122: 2063.
10. Lang, P. *et al.* (2013) Blood 122: 692.

MACS® Technology – meeting the challenge of isolating cells with high purity, gently and rapidly

In the 1980s, cell separation methods were based mostly on the cells' physical or biochemical properties, for example, density, size, the sensitivity towards certain compounds, or the capability to stick to certain surfaces. These techniques have major drawbacks, such as low specificity and sensitivity, poor separation results, and low cell yield. Moreover, these methods do not allow for the isolation of particular cell subsets.

Fluorescence-activated cell sorting was (and still is) a powerful technique for the isolation

of cells at high purity based on multiple cell markers. However, flow sorting requires complex instrumentation and allows only low cell throughput, making it particularly difficult to isolate rare cell types in a reasonable amount of time. In addition, the conventional flow sorting technique is inherently stressful to the cells.

Facing these challenges Stefan Miltenyi developed MACS® Technology, a novel cell separation technology based on MicroBeads – small nanosized paramagnetic particles bound to specific monoclonal antibodies. This marked the beginning of Miltenyi Biotec in 1989. MACS Technology allows for the gentle magnetic separation of large amounts of cells with high purity and yields – in no time and with minimal equipment. Due to its compelling benefits, the technology quickly became the gold standard in cell separation – cited in more than 20,000 publications to date.

The CliniMACS® System – enabling translation into clinical settings

Right from the start, Miltenyi Biotec's goal was to provide tools for basic research as well as for translation of the findings into clinical application to advance cellular therapy. Consequently, the CliniMACS® System was developed in 1997 based on MACS Technology, enabling the clinical-scale enrichment of CD34$^+$ hematopoietic stem and precursor cells in a closed system. Continuous research towards a cell therapy based on enriched CD34$^+$ cells culminated in the recent FDA approval of the CliniMACS CD34 Reagent System (info box on p. 6).

More and more reagents for the enrichment of other cell types have been developed since 1997. With its great versatility, the CliniMACS System now provides the basis to explore a wide range of cell therapy options, including i) enrichment of CD34$^+$ cells and depletion of T cell subsets and B cells from HSCT grafts, ii) manufacture of T cell subsets and NK cells for donor lymphocyte infusions towards the development of therapies of several types of cancer, iii) enrichment of CD133$^+$ precursor cells and CD271$^+$ mesenchymal stromal cells for tissue regeneration, and iv) the manufacture of dendritic cells (DC) for the generation of DC-based vaccines, either by direct positive selection of CD304$^+$ plasmacytoid DCs or enrichment of monocytes, which can subsequently be differentiated into DCs.

The CliniMACS System has major advantages over flow sorting, including higher cell throughput, higher cell recovery, more gentle cell processing, less hands-on time, and lower processing costs per cell dose. Moreover, the CliniMACS System is compatible with standard blood bank workflows as cell separation occurs in a sterile, closed system. The CliniMACS System, including the CliniMACS Plus Instrument, provides for a high level of automation in cell enrichment. However, some cell manufacturing processes are highly complex and include a number of steps in addition to cell separation, thus still requiring considerable hands-on time. These processes include, for example, the enrichment of antigen-specific T cells based on their cytokine secretion properties or the manufacture of T cells expressing chimeric antigen receptors (CARs). To automate even most complex cell manufacturing procedures, Miltenyi Biotec developed the CliniMACS Prodigy®.

The CliniMACS Prodigy® – mastering the complexity of cell processing

The CliniMACS Prodigy® integrates all cell processing steps, including sample preparation, cell washing, density gradient centrifugation, magnetic cell separation, cell activation, genetic modification, cell culture, and final cell product formulation (fig. 1). The fully automated, sensor-controlled processes provide for a high level of standardization and reproducibility. Hands-on time is reduced substantially. As all steps are performed in single-use, closed tubing sets, the instrument also reduces cleanroom requirements. In combination with the wide variety of GMP Products manufactured by Miltenyi Biotec, the CliniMACS Prodigy facilitates the implementation of GMP-compliant cell processing.

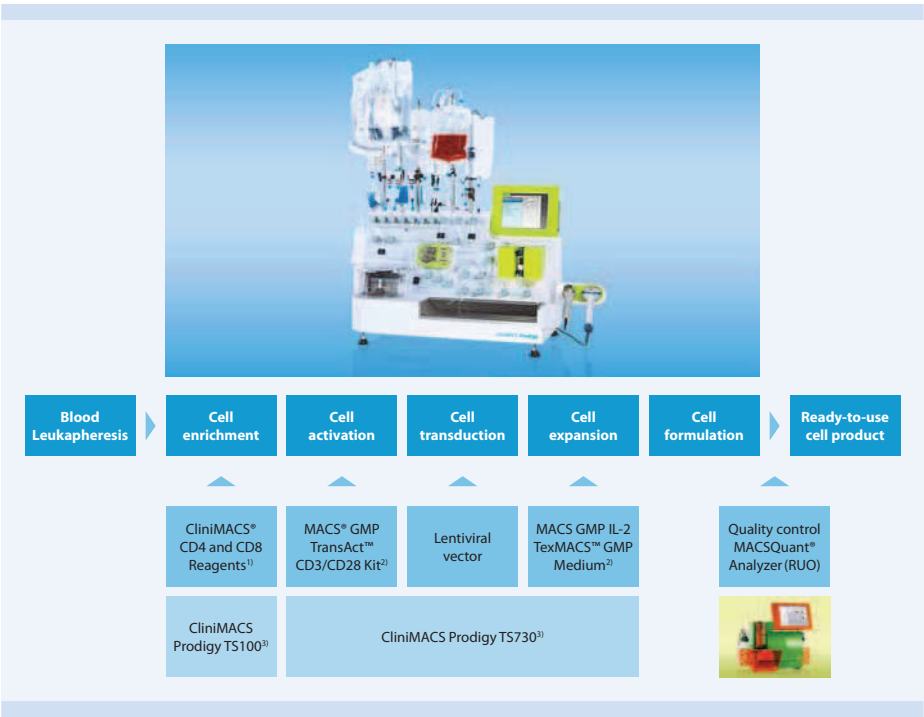


Figure 1: The CliniMACS Prodigy and dedicated tubing sets enable complex automated workflows in an enclosed system. The scheme exemplifies the process for the manufacture of CAR T cells. All indicated steps are performed on the CliniMACS Prodigy with tubing sets CliniMACS Prodigy TS100 and TS730. Miltenyi Biotec offers numerous GMP Products for cell processing. The broad portfolio of flow cytometry tools, including powerful MACSQuant® Flow Cytometers and hundreds of antibodies, allows for a detailed cell analysis in translational research.

[1] In the USA, the CliniMACS CD4 and CD8 Reagents are available for use only under an approved Investigational New Drug (IND) application or Investigational Device Exemption (IDE). [2] MACS GMP Products are for research use and *ex vivo* cell culture processing only, and are not intended for human *in vivo* applications. [3] In the USA, the components of the CliniMACS Prodigy System are for research use only.

Occasionally, the enrichment of complex cell subsets requires sophisticated sorting strategies based on multiple markers, going beyond the capabilites of magnetic cell separation. To complement magnetic cell separation with the option of multiparameter, high-speed flow sorting, Miltenyi Biotec recently developed the MACSQuant® Tyto™.

The MACSQuant® Tyto™ – microchip technology revolutionizing cell sorting

The MACSQuant® Tyto™ enables 11-parameter fluorescence-based cell sorting. All sorting processes occur in a fully enclosed, sterile cartridge, thus facilitating the translation into clinical applications. High-speed flow sorting with the MACSQuant Tyto (figs. 2 and 3) is made possible by the world's fastest mechanical sort valve, based on microchip technology. The instrument enables sorting speeds of up to 108,000,000 cells per hour. Conventional flow sorting involves sheath fluids and the formation of droplets and aerosols. In contrast, the MACSQuant Tyto does not use a nozzle. Instead, it drives the cells through the microchip with low positive pressure. This results in less stress to the cells and ultimately in a higher cell yield and viability. The patented cartridge system keeps samples free from contaminations and prevents exposure of the operator to potentially harmful sample material. The cartridge system is easy to operate in an intuitive plug-and-play fashion and does not require specialized technical expertise.

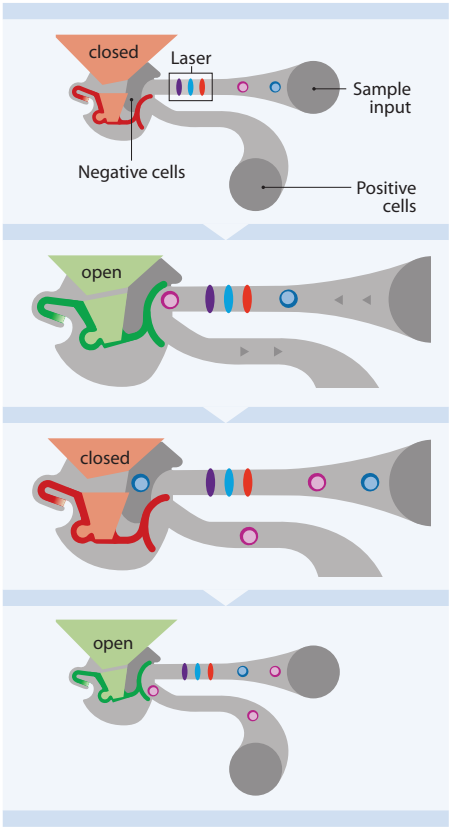


Figure 2: Mechanism of microchip-based sorting. Labeled cell samples enter the chip from the sample input area. As the cells approach the sort area, each cell is analyzed. When a selected cell is identified, a magnetic pulse opens the valve and the cell is redirected to a collection chamber. An integrated single-crystal silicon spring returns the valve to its original position, and undesired cells are allowed to flow through.

Figure 3: The MACSQuant Tyto and proprietary cartridges allow for gentle, 11-parameter fluorescence-based cell sorting in an enclosed system.

A whole new world of clinical-scale cell manufacture

The combination of CliniMACS Prodigy and MACSQuant Tyto will open up a whole new world of clinical-scale cell manufacture in the future. Automated cell processing on the CliniMACS Prodigy along with microchip-based cell sorting on the MACSQuant Tyto provide the potential to enrich even most demanding, rare cell subsets – with minimal hands-on time. Starting from up to 10^{11} cells, the CliniMACS Prodigy enables the pre-enrichment of a particular cell population. Subsequently, the target cells can be further purified according to multiple markers with the MACSQuant Tyto. Further cell processing, such as genetic modification, cell expansion, and formulation of the final cell product can then be performed automatically on the CliniMACS Prodigy.

25 years of development and innovation at Miltenyi Biotec have raised clinical-scale cell processing to new heights, offering tremendous opportunities for the development of sophisticated cellular therapies.

A leap forward in cancer research

Many cancer researchers rely on human tumor xenograft models to mimic *in vivo* the patient tumor. However, contaminating mouse cells frequently hamper analysis of the real target cells. Miltenyi Biotec provides a complete solution for effective mouse cell depletion, allowing for an accurate, unbiased xenograft analysis.

Xenotransplantation in cancer research

Xenograft technology facilitates understanding tumor biology, supports drug discovery, and has opened the door to personalized medicine. Xenografts consist of primary human tumor tissue, serially transplanted tumor tissue, or cultured tumor cells. When the xenograft is transplanted into mice, it is vascularized and infiltrated by mouse cells, including heterogeneous lymphocyte populations, fibroblasts, and endothelial cells. The level of infiltration depends on a number of factors, such as the type of tumor, growth rate, and region of transplantation. Even when these factors are kept constant, the amount and composition of infiltrating mouse cells are highly variable. After a certain growth period, the xenograft, including the infiltrating mouse cells, is removed from the host and the tumors are analyzed by cellular or molecular assays. However, in many analytical methods the contaminating mouse cells pose the risk of bias, leading to erroneous conclusions on the properties of the tumor cells.

Tumor xenograft models – great potential, substantial limitations

Xenotransplantation of human tumor tissues or cells is a widespread technique in cancer research. Commonly, the xenograft-derived cells are analyzed on a molecular level using microarrays, next-generation sequencing, or proteomic approaches.

On the one hand, microarrays allow for the sensitive detection of even minute molecular changes within a cell population. On the other hand, the technique is also susceptible to results derived from contaminating mouse cells due to cross-hybridization of mouse-derived molecules to human probes. Moreover, the sensitivity of next-generation sequencing and proteomic analysis is significantly reduced due to signals originating from mouse cells. Xenograft-derived cells can also be used to establish cell lines in culture. However, culturing the human tumor cells is often hampered, as particularly mouse fibroblasts tend to overgrow the cultures.

Many researchers try to avoid these drawbacks by depleting CD45$^+$ and MHCI$^+$ cells from the xenografts. However, antibodies against the two markers label only a subfraction of mouse cells, and therefore do not allow for a complete removal of these cells. Positive selection of CD326 (EpCAM)$^+$ tumor cells is not a good option either, as this marker is not expressed on all tumor cells. Moreover, even in tumors that are generally EpCAM-positive, subpopulations of tumor cells down-regulate EpCAM expression during epithelial-to-mesenchymal transition.

Depletion of all mouse cells is the key to uncover the full potential of tumor xenografts

Miltenyi Biotec researchers recognized the possibility of harnessing the full potential of tumor xenograft models. They set out to develop a tool that would enable the depletion of all mouse cells for an unbiased analysis and clean cultures of tumor cells.

"During our visits in cancer research labs, scientists were thrilled when we presented the idea of working with pure human xenograft-derived cancer cells – and we visited quite a few labs across the globe, for example, the NIH, MD Anderson Cancer Center, and the Dana-Farber Cancer Institute in the U.S., Institut Curie and IGR in Paris, the CNIO in Madrid, and the DKFZ in Heidelberg," recalled Dr. Christoph Hintzen, Product Manager at Miltenyi Biotec.

The development began with an elaborate screening of 150 antibodies to find the right combination of surface markers that would allow for the detection and removal of all mouse cells across multiple organs. Finally, the screening resulted in five mouse-specific markers for effective labeling and depletion of all mouse cells (fig. 1). The appropriate antibodies conjugated to MACS® MicroBeads, combined in a single Mouse Cell Depletion Cocktail, are now part of the Mouse Cell Depletion Kit.

The complete solution for separating human tumor xenografts from mouse cells

"The cell surface epitopes that are required for effective mouse cell depletion are highly sensitive to degradation during enzymatic tissue dissociation. Therefore, it is crucial to use highly pure enzymes and an optimized process for reliable cell preparation," Dr. Olaf Hardt, Senior Project Manager, R&D, at Miltenyi Biotec, explained. Comprehensive research and development efforts resulted in an integrated solution for mouse cell depletion from xenografts. "With the Tumor Dissociation Kit, human and the gentleMACS™ Octo Dissociator with Heaters all required epitopes are preserved during tissue dissociation, so that the Mouse Cell Depletion Cocktail effectively removes all mouse cells," Olaf Hardt continued.

Mouse cell depletion can be performed manually with individual xenograft samples, using MACS Separators and Columns. However, xenograft studies often require parallel processing of samples from multiple mice. In this case, the MultiMACS™ Cell24 Separator Plus simplifies the depletion process as it enables automated cell separation from up to 24 samples in a single run. The isolated

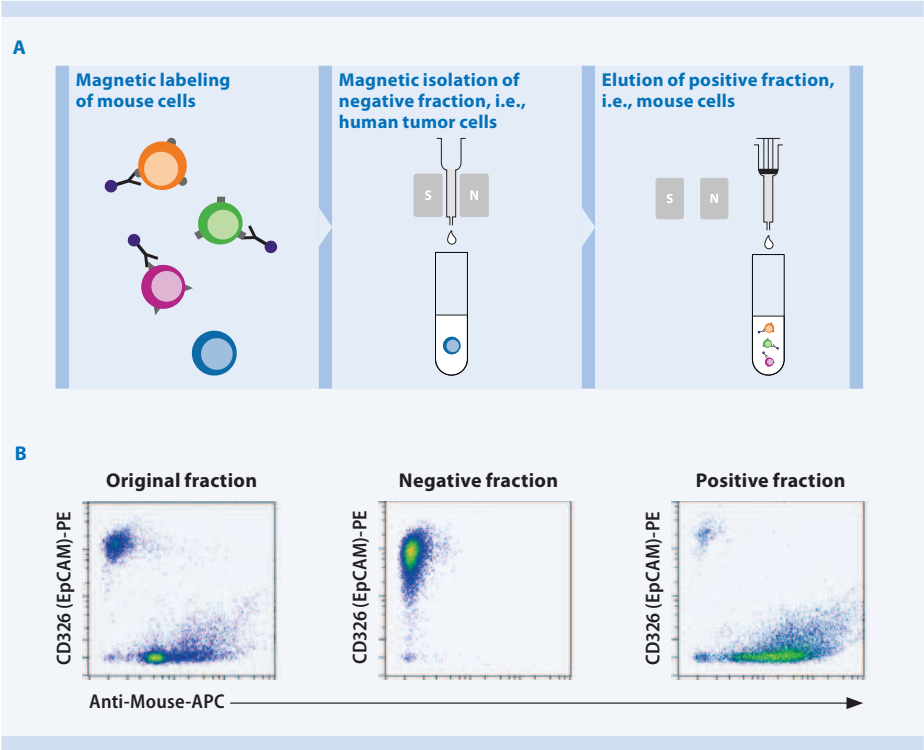


Figure 1: Mouse cell depletion from human tumor xenografts. (A) Principle of mouse cell depletion. (B) Mouse cell depletion from a CD326 (EpCAM)$^+$ human tumor xenograft. The tumor xenograft was dissociated using the Tumor Dissociation Kit and the gentleMACS Octo Dissociator with Heaters. The resulting single-cell suspension was separated into human tumor cells and mouse cells using the Mouse Cell Depletion Kit. Before and after separation cells were labeled with CD326 (EpCAM)-PE and APC-conjugated versions of the five antibodies (Anti-Mouse-APC) used in the Mouse Cell Depletion Kit. Cells were analyzed with a MACSQuant® Flow Cytometer.

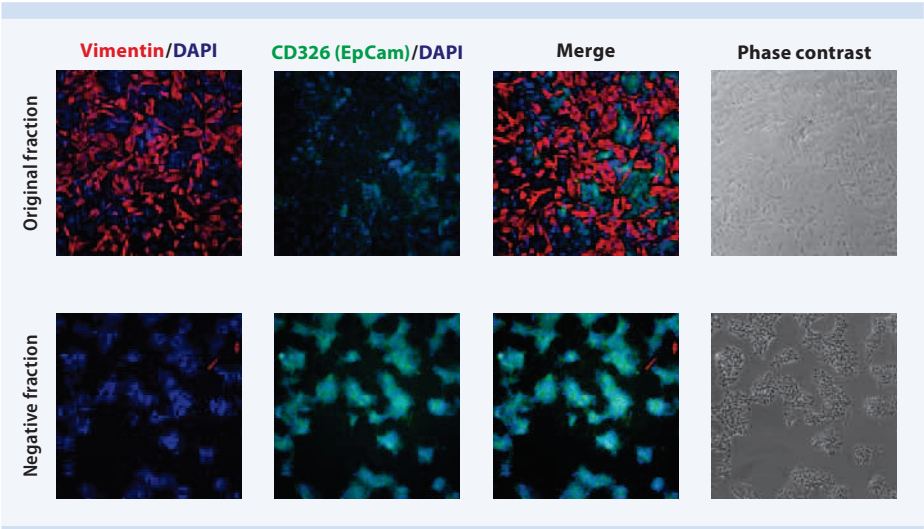


Figure 2: Cultures of dissociated tumor xenograft cells. The tumor xenograft was dissociated using the Tumor Dissociation Kit and the gentleMACS Octo Dissociator with Heaters. The resulting single-cell suspensions were either left unseparated (upper row) or were depleted of mouse cells using the Mouse Cell Depletion Kit (lower row). The cell fractions were cultured for three days prior to analysis by immunofluorescence microscopy. The vimentin antibody labels fibroblasts, the CD326 (EpCAM) antibody labels tumor cells.

human tumor cells are unlabeled and can be used for any application, including molecular analyses, flow cytometry, isolation of tumor cell subpopulations, and cell culture (fig. 2). Miltenyi Biotec offers a variety of products for downstream applications of the human tumor cells, including flow cytometry antibodies against CD326 (aka EpCAM, a marker for some tumor cells) and many cancer stem cell (CSC) markers. For research groups wanting to further characterize subpopulations within the human cell fraction, MACS Cell Separation Reagents are available for the isolation of tumor cells based on CD326 (EpCAM) and ErbB-2, or CSCs based on CD44, CD24, CD133, Lgr5, and other markers.

"The impact of mouse cell removal on experimental results is tremendous and very easy and fast to accomplish. Including tumor dissociation it takes a little over an hour to obtain pure tumor cells. I can't imagine why anyone would miss the opportunity to eliminate artifacts caused by the contaminating mouse cells," Jacqueline Sutter, Product Manager at Miltenyi Biotec, pointed out.

The Mouse Cell Depletion Kit

- Fast isolation of human tumor cells from dissociated xenografts (<20 min)
- Easy-to-perform procedure
- Applicable to all kinds of xenografted human tissues or cells, as no human–specific marker is required
- Optimized automated tumor dissociation process available, ensuring best possible mouse cell removal
- Automation available for processing of up to 24 samples in parallel

For more information visit
www.miltenyibiotec.com/xenograft

An alliance with Miltenyi Biotec in the fight for tolerance: A venture into no man's land



Niloufar Safinia, Giovanna Lombardi, and Robert Lechler
Immunoregulation laboratory, MRC Centre for Transplantation, King's College London, Guy's Hospital, London, UK

Over the past few years a close partnership with Miltenyi Biotec has led to the successful development of technologies required for production of GMP-grade, functionally suppressive human regulatory T cells at clinically applicable numbers, from both healthy donors and patients (ref. 1, and Fraser and Safinia, manuscript in preparation).

The work highlighted here represents a high-impact translational cell therapy program, showcasing our state-of-the-art GMP-compliant Cell Therapy Core, Cell Sorting Facility, and Clinical Research Facilities. The coming together of a critical mass of translational scientists within this program, supported by Miltenyi Biotec, and a substantial institutional commitment has granted us a unique opportunity to translate the findings from our own high-impact scientific work into a novel, clinically primed treatment for patients on the transplant waiting list.

Introduction

Transplantation is currently the only effective treatment for end-stage organ failure. However, its promise as a successful treatment modality is severely hampered by the requirement for long-term use of immunosuppressants, associated with significant morbidity and toxic side effects. The panacea that is 'tolerance induction' will enable the minimization or complete withdrawal of these immunosuppressive drugs thus negating any long-term toxic sequelae.

Many different strategies aimed at inducing tolerance have been proposed to date, including: T cell/cytokine modulation [2], co-stimulation blockade [3], peptide-based immunotherapy [4], and microbiome modulation [5]. Over the last 5 years, there has also been a dramatic growth in the cell therapy industry, opening up a new avenue in the pursuit of tolerance. In parallel, our own research has focused on the prospects of adoptive cell therapy with regulatory T cells (Tregs) to induce tolerance in the setting of solid organ transplantation.

$CD4^+CD25^+FOXP3^+$ Tregs have been described as key regulators in various immunological processes and are ideal candidates for therapeutic strategies aimed at tolerance induction. Animal studies, from our lab and others, have provided evidence in support of the feasibility and effectiveness of such a therapy. In this regard, we have previously shown that infusion of recipient murine Tregs (expanded *in vitro*) can prolong skin allograft survival and induce the indefinite acceptance of heart allografts [6]. Moreover, using humanized mouse models of skin and islet cell transplantation, we have shown that the adoptive transfer of polyclonal human Tregs protects against alloimmune-mediated skin pathology [7] and results in the increased survival of transplanted islets [8].

In the last 5 years, results of the first clinical trials with Tregs have been published, supporting the safety and potential efficacy of Treg adoptive cell therapy in preventing/treating graft-versus-host disease (GVHD) [9-11]. Another recently published study demonstrated that autologous Treg therapy could be safely used to delay the onset of type I diabetes in children by averting islet β-cell destruction [12]. The clinical data to date is encouraging and supports the rationale that Treg adoptive transfer is likely to be a safe and successful strategy for inducing transplantation tolerance, whilst reducing the requirement for immunosuppression. Indeed, we are taking *ex vivo* expanded Tregs forward to clinical trials of our own:

1. To prevent renal transplant rejection, we are pursuing a trial as part of an EU FP7 consortium, the 'ONE Study'.
2. To prevent liver transplant rejection, we initiated 'ThRIL', a dose escalation study, where we will assess the safety and tolerability of polyclonally expanded Tregs in combination with depletion of alloreactive T cells and short-term immunosuppression.

Our quest to devise a clinically applicable protocol for tolerance induction, using polyclonally expanded Tregs, has been especially challenging, fundamentally since the number of these cells in peripheral blood is low and considerable numbers of Tregs are needed for the prevention of graft rejection [13]. However, the recent developments and technical improvements in GMP-compliant Treg isolation and *ex vivo* expansion products, provided by Miltenyi Biotec, has enabled us to make further progress along the path towards achieving clinical tolerance. In a previous publication [1] we successfully expanded human Tregs from patients with end-stage kidney disease (ESKD) in the laboratory, using

GMP-compatible reagents and protocols, as prelude for the ONE Study. Here we present the most recent data on the validation of our GMP-compliant Treg expansion protocol in the GMP Clinical Research Facility (CRF) at Guy's Hospital and address its translation to the clinic.

Methods

Treg isolation and *ex vivo* expansion

200 mL of blood was obtained from two patients with ESKD on hemodialysis, after informed consent (Institutional Review Board approval 09/H0707/86), and 1 unit of blood from a healthy subject from NHSBT (NHS Blood and Transplant). Patient exclusion criteria included: recent illness (within the previous two months), significant anemia, autoimmune disease, and previous transplants. Blood volume was reduced using the Sepax® 2 device (Biosafe) prior to Treg isolation.

The clinical Treg isolation protocol involved a combination of $CD8^+$ cell depletion and a positive selection step for $CD25^+$ cells, with the isolation tool mainly involving the automated CliniMACS® Plus System (Miltenyi Biotec). All reagents and consumables used were of GMP grade and processing steps were performed in closed systems, using bags.

Enriched cells were seeded in MACS® GMP Cell Expansion Bags at 0.5×10^6 cells/mL in TexMACS™ GMP Medium (Miltenyi Biotec) supplemented with 5% human serum, containing 100 nM rapamycin (Rapamune®). Cells were activated with anti-CD3– and anti-CD28–coated beads (4:1 bead:cell ratio, MACS GMP ExpAct Treg Kit, Miltenyi Biotec). Human recombinant IL-2 (500 IU/mL; Proleukin®) was added at day 4–6 and replenished every 2–3 days. The cells were rested 4 days before restimulation. Stimulation occurred on days 12 and 24, during which time cells were pooled, fresh beads (1:1), rapamycin, and IL-2 added and the suspension seeded into bags (250, 500, or 1000 mL). For a schematic representation of the protocol see figure 1. Expanded cells were harvested on day 36. The pooled cells were run on the CliniMACS Instrument using a pre-set program for depletion to remove the ExpAct Treg expansion beads to form a bead-depleted cell population. A small aliquot of the cells was then taken for safety and functional analysis.

Phenotype and functional analysis of the isolated and expanded cells

Phenotype: Cell surface and intracellular markers were analyzed to confirm the identity and purity of the freshly isolated and expanded cells. Cells were labeled using antibodies against the cell surface markers (CD4-PerCP/Cy™5.5, CD25-PE, CD8-APC) and an intracellular marker (FOXP3-FITC). Appropriate isotype controls and fluorescence-minus-one controls were used to assign gates. Analysis was carried out using the FlowJo® software (Treestar).

Function: Cryopreserved $CD4^+CD25^-$ T cells (Teff) were thawed and labeled with 2.5 nM CFSE and cultured alone or with Tregs at Treg:Teff ratios of 1:1, 1:5, and 1:10. Cells were activated by anti-CD3/CD28–coated beads and cultured for 5 days. After harvest, proliferation of CFSE-labeled Teffs was assessed by flow cytometry and the data analyzed subsequently using the FlowJo software. The suppressive ability of Treg lines was quantified by the percentage decrease of Teff proliferation in the presence of Tregs. The calculation was based on the proliferation of Teffs alone compared with the proliferation of cultures containing Teffs and Tregs. All batches had to fulfill the set release criteria that included: i) $CD4^+CD25^+FOXP3^+$ cells ≥ 60% of entire cell population; ii) $CD8^+$ cells ≤ 10%; iii) ≤100 beads per 3×10^6 cells; iv) viability ≥ 70%; v) sterility: no growth after 5 days; vi) endotoxin ≤ 175 IU/mL; vii) mycoplasma: not detected; viii) suppression ≥ 60% (fig. 1).

Cryopreservation of the expanded Tregs

After final harvest, cells were centrifuged, supernatant removed, and the cells resuspended in the required volume of

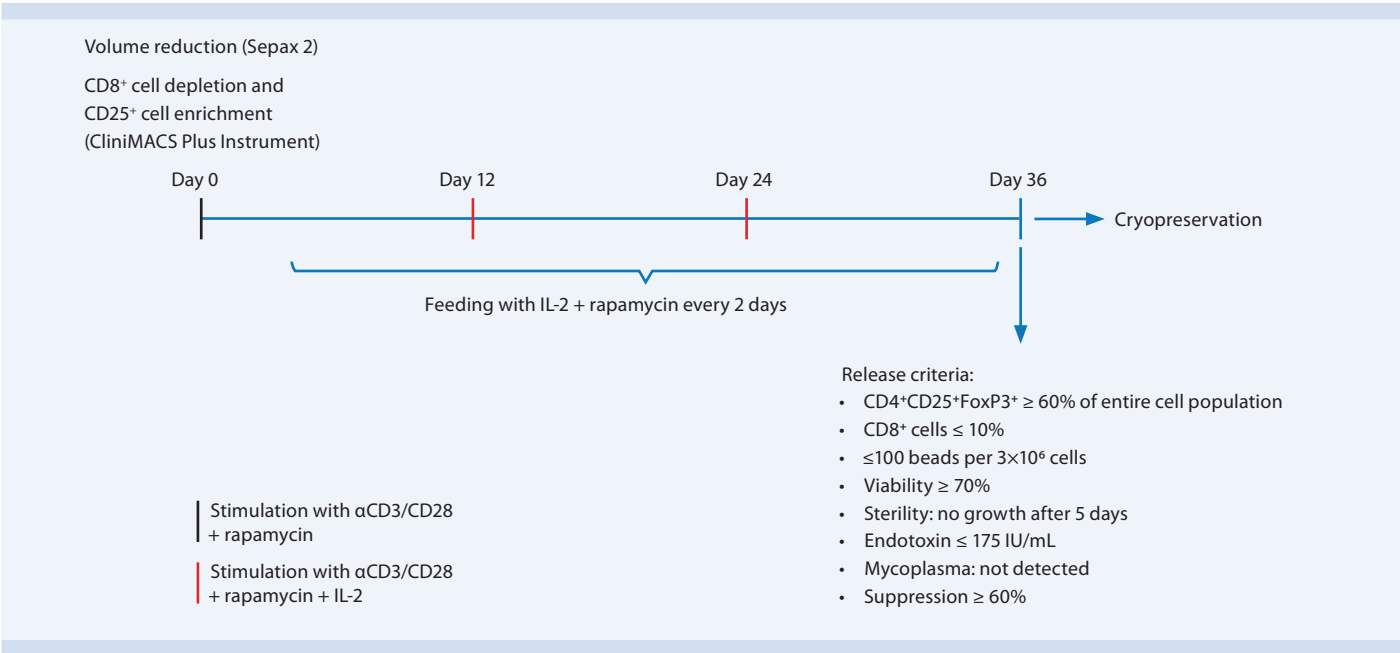


Figure 1: Schematic representation of the GMP-compliant protocol for Treg isolation and expansion

CryoStor® CS10 freezing media. The product was stored in CellSeal® Cryovials and placed in a controlled rate freezer and subsequently transferred to liquid nitrogen (vapor phase) for long-term storage.

In order to assess the recovery of the cryopreserved product and the effect of cryopreservation on the biology and function of the final product, cells were thawed, diluted in 5% human serum albumin, and the viability and suppressive function of the cryopreserved product was determined. Cell viability was tested by the trypan blue exclusion test with concurrent assessment of suppressive function (described above).

Results and discussion

Tregs can be expanded *in vitro* under GMP conditions to yield clinically relevant numbers

One of the major obstacles to Treg cellular therapy has been the generation of sufficient numbers of cells to maximize efficacy. The required Treg number is unknown, however the aim is to increase the Treg:Teff ratio, with data from animal models suggesting a ratio of Treg:Teff at 1:1 and 1:2 to be optimal [13].

In a previous publication [1] we carried out an in-depth characterization of Tregs isolated from patients with ESKD as compared to healthy donors (control). From this work we were able to show that these patients and healthy donors have similar numbers of Tregs (5.2% ± 2.0% compared with 4.6% ± 1.7% for healthy donors and ESKD patients, respectively; p = 0.41). While it is not only desirable to achieve a sufficient starting number of Tregs, the expansion profile of Tregs from patients should also be comparable to that of cells from healthy donors to ensure a successful expansion. Here, we sought to isolate Tregs using the CliniMACS System and compared the recovery of the isolated cells between patients and healthy donors. Despite an initial lower recovery of isolated Tregs from patients (1×10^6 and 0.46×10^6) as compared to healthy donors (5×10^6), partly explained by the amount of the starting material used, the expansion profile was comparable between the two groups, with Tregs expanding to numbers suitable for their clinical application (table 1).

Ex vivo expanded Tregs from patients and healthy donors yield an enriched population, which is functionally suppressive, achieving the release criteria needed for their clinical application

A comprehensive phenotypic characterization of the expanded Tregs was performed to ensure the final product satisfied the specified release criteria in order to allow their future clinical application. Viability was assessed by trypan blue exclusion with cells stained for CD4, CD25, and the intracellular transcription factor, FOXP3. Flow cytometric analysis of the Tregs at final harvest concluded that the percentage of cells with a $CD4^+CD25^+FOXP3^+$ phenotype was 88.2% for the healthy donor and 74.7% and 76.7% for the patients. The viability of the final product was >95%. With >80% suppressive function, the expanded Tregs from both groups exhibited potent suppressor activity in the classical suppression assay. In addition, contamination with $CD8^+$ cells was minimal (<10% of $CD8^+$ cells in both groups) and all final products passed the necessary sterility tests as defined in the release criteria (table 2).

The data summarized above is promising, demonstrating that we are able to expand sufficient numbers of Tregs from patients with ESKD whilst ensuring the maintenance of a functionally pure and suppressive population. However, for the clinical trials planned, cryopreservation is required to accommodate for the adoptive transfer of these cells at different time points, i.e., at 5 days and 3 months post transplantation for the ONE study and ThRIL study respectively. In addition, the concept of cryopreserving the final product will allow more flexibility in terms of the timing of the infusion, holding the possibility of administering multiple infusions in future trials. However, current knowledge of how the process of cryopreservation may affect Tregs is still limited. The data summarized below focuses on our findings 12 weeks after Treg cryopreservation, assessing the effects of the freeze/thaw process on the expanded cells with regard to their biology and function. We are also currently conducting further experiments on the cryopreserved product to ensure stability of Tregs at different time points after cryopreservation (data not shown).

Cryopreservation of expanded Tregs

Current experience with Treg cryopreservation is limited. Published studies differ in freeze/thaw techniques, either storing the isolated Tregs after leukapheresis, weeks/months before the transplantation [14] or, as in the clinical trial of Tregs isolated from umbilical cord blood (UCB) for the treatment of GVHD, storing the cells after *ex vivo* expansion [11]. Choice of an appropriate protocol for cryopreservation of Tregs plays a critical role in achieving a high recovery of fully functional Tregs after cryopreservation. Various different factors have been studied, leading to improvements in cryopreservation technique [15-17]. The method we have used, outlined above, resulted in a viability of >75% for both the patient Treg cultures and healthy controls, when cells were thawed 12 weeks after cryopreservation. In addition, Treg recovery amounted to >90% for both groups with maintenance of phenotype, as assessed by intracellular staining of FOXP3 as well as the surface markers CD4 and CD25. Of importance, the thawed cells had maintained their suppressive function with >80% suppressive capability of Tregs from patients and healthy donors (table 2). This data is reassuring in view of the clinical application of cryopreserved Tregs.

Donor	Total cells $\times10^6$			
	Stim 1 (Day 0)	Stim 2 (Day 12)	Stim 3 (Day 24)	Final harvest (Day 36)
ESKD 1	0.46	4.5	32	116.4
ESKD 2	1	21	193	770
Healthy donor	5	17.5	79.2	303

Table 1: Clinical-grade Tregs expanded from blood samples of ESKD patients and a healthy donor have similar expansion profiles, reaching numbers suitable for clinical application. The different recovery at day 0 between patients and healthy donor is due to differences in the amount of sample obtained, 200 mL from patients and 1 unit of blood from NHSBT for the healthy donor.

Release criteria	Specification	Healthy donor		ESKD 1		ESKD 2	
		Final harvest	Defrosting	Final harvest	Defrosting	Final harvest	Defrosting
Identity	Positive for CD4, CD25, FoxP3	Yes	Yes	Yes	Yes	Yes	Yes
Purity (%)	$CD4^+CD25^+FoxP3^+$cells ≥ 60% of entire cell population	88.2	71.7	74.7	70.5	76.7	88.6
Impurity (%)	$CD8^+$ cells ≤10%	0.41	4.68	1.82	1.69	1.6	3.26
Viability (%)	Viability ≥70%	96.5	76	96	93	95	82
Recovery (%)	≥70%		97		93		110
Potency (%)	Suppression ≥ 60%	81	97	83.1	84	91.3	95
Safety tests	Sterility: no growth	Pass		Pass		Pass	Pass
	Endotoxin: ≤175 IU/mL	<5 IU/mL		<50 IU/mL		<50 IU/mL	<5 IU/mL
	Mycoplasma: not detected			Not detected		Not detected	Not detected

Table 2: Data on the final harvest, showing that cells met the necessary release criteria on day 36. At 12 weeks after cryopreservation cells maintained their phenotype and suppressive function.

Conclusion

The potential of using Tregs as a cellular clinical therapy is rapidly becoming a reality. Here we present data on the validation of our protocol (manuscript in preparation) in our GMP Clinical Research Facility. We have shown that Tregs from both patients and healthy donors can be expanded, after isolation using the CliniMACS System, to numbers suitable for their clinical application. Furthermore, the cells maintain their phenotype and function throughout the 36-day culture period and fulfill the release criteria set for our clinical trials. With regard to the cryopreservation of the cells, we have clearly demonstrated that following the freeze/thaw process Treg viability, phenotype, and suppressive function are maintained. As a result, we are now only a few steps away from the clinical application of our product in phase I studies in the setting of solid organ transplantation.

Future perspective

In view of animal data from our laboratory[7,18] and others in support of the importance of antigen-specific Tregs in the setting of solid organ transplantation, we are currently developing GMP-compatible protocols for the generation of Tregs with direct allospecificity. The delineation of an optimal antigen-presenting cell for the allospecific *ex vivo* expansion of Tregs is at the heart of the debate, with recent studies indicating that allogeneic B cells may be highly effective[19,20]. Our initial data has highlighted that a highly pure population of Tregs prior to allospecific *ex vivo* expansion is essential for this to be a success. With the imminent installation of a GMP-compliant cell sorter into our facilities, efforts will be directed towards the generation of an optimal precursor population of antigen-specific Tregs for cellular therapy in the near future.

References

1. Afzali, B. *et al.* (2013) Clin. J. Am. Soc. Nephrol. 8: 1396–1405.
2. Zhang, X. *et al.* (2012) Transpl. Immunol. 27: 63–68.
3. Sandborn, W.J. *et al.* (2012) Gastroenterology 143: 62–69.
4. Kim, E.H. (2011) J. Allergy Clin. Immunol. 127: 640–646.
5. Atarashi, K. and Honda K. (2011) Curr. Opin. Immunol. 23: 761–768.
6. Tsang, J.Y. *et al.* (2008) J. Clin. Invest. 118: 3619–3628.
7. Sagoo, P. *et al.* (2011) Sci. Transl. Med. 3: 83ra42.
8. Xiao, F. *et al.* (2014) PloS One 9: e90387.
9. Trzonkowski, P. *et al.* (2009) Clin. Immunol. 133: 22–26.
10. Di Ianni, M. *et al.* (2011) Blood 117: 3921–3928.
11. Brunstein, C.G. *et al.* (2011) Blood 117: 1061–1070.
12. Marek-Trzonkowska, N. *et al.* (2012) Diabetes Care 35: 1817–1820.
13. Tang, Q and Lee, K. (2012) Curr. Opin. Organ Transplant. 17: 349–354.
14. Peters, J.H. *et al.* (2008) PloS One 3: e3161.
15. Weinberg, A. *et al.* (2009) Clin. Vaccine Immunol. 16: 1176–1186.
16. Brooks-Worrell, B. *et al.* (2011) Diabetes Metab. Res. Rev. 27: 737–745.
17. Mallone, R. *et al.* (2011) Clin. Exp. Immunol. 163: 33–49.
18. Putnam, A.L. *et al.* (2013) Am. J. Transplant. 13: 3010–3020.
19. Zheng, J. *et al.* (2010) Cell. Mol. Immunol. 7: 44–50.
20. Tu, W. *et al.* (2008) Blood 112: 2554–2562.

Acknowledgments

The authors thank all the research subjects and patients who volunteered for this study and acknowledge the hard work and dedication of the GMP scientists; Dr Henrieta Fraser, Sarah Thirkell, and Katie Lowe in obtaining the data presented in this manuscript. A special thank you to Sarah Thirkell for also reviewing the data presented and to Chris Fisher, head of GMP production and Drew Hope, head of GMP quality. This research was supported by the ONE Study, the Medical Research Council (MRC) Centre for Transplantation, King's College London, UK – MRC grant no. MR/J006742/1 and the National Institute for Health Research (NIHR) Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

NK cell transplantation as consolidation therapy in children with acute myeloid leukemia – results from a pilot study

Wing Leung
Department of Bone Marrow Transplantation and Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN, USA

Introduction

Acute myeloid leukemia (AML) is the second most common form of acute leukemia in children. Despite intensive chemotherapy, approximately half of the AML patients will suffer a relapse. High-dose chemotherapy with autologous stem cell transplantation fails to improve patient outcomes.[1] Several cooperative group studies have demonstrated that allogeneic bone marrow transplantation for AML in first complete remission with HLA-identical sibling donor yielded favorable results when compared with chemotherapy without transplantation.[1] However, bone marrow transplantation is expensive and is associated with many acute and late complications.[2,3] Furthermore, the majority of patients do not have a matched sibling donor.

Natural killer (NK) cells are a type of normal lymphocytes, along with B and T cells. As a component of the innate immune system NK cells play an important role in early defense against infections. They may also interact with antigen-presenting cells to shape the adaptive immunity. A large epidemiological study has clearly defined the crucial role of NK cells in cancer surveillance.[4] Many patients with leukemia have an NK cell deficiency after completion of standard therapy.[5] Thus, transplantation of NK cells from normal donors appears to be an attractive means to restore NK immunity against cancer recurrence.

NK cell activity is controlled by a receptor gene family called killer cell immunoglobulin (Ig)-like receptors (KIRs).[6] There are 15 KIR genes (plus two pseudogenes) and the gene name is based on structure (number of Ig-like domains and length of tail). Eleven genes encode receptors with two Ig-like domains, four with three Ig-like domains, nine with long tail and six with short tail. KIRs with long tail contain immunoreceptor tyrosine-based motifs (ITIM) that may function as an inhibitory receptor, whereas those with short tail may interact with DAP12 to participate in activation signaling. KIRs show an extreme diversity in the human genome (similar to HLA), in terms of gene content, expression level, and allelic polymorphism.[7,8] Therefore, no individual possesses all possible KIR genes and alleles. Thus, transplantation of NK cells from a donor who has a certain KIR gene that is absent in the recipient may be considered as a form of "natural gene therapy".

We have previously shown that in haploidentical stem cell transplantation for leukemia, a low relapse rate was associated with the presence of inhibitory KIRs in the donor without a cognate HLA ligand in the recipient (i.e., donor-recipient receptor-ligand mismatch).[6] Therefore, we hypothesized that transplantation of NK cells from a receptor-ligand mismatched donor as consolidation therapy might prevent a leukemia relapse. Our first application was on childhood AML, as many patients would be expected to show a leukemia relapse after completion of chemotherapy. A summary of this study[9] is described here.

Patients and methods

Ten pediatric patients with low- or intermediate-risk AML were enrolled[9] (table 1). High-risk patients received allogeneic bone marrow transplantation as consolidation therapy instead. All of the participants have completed four or five cycles of standard intensive chemotherapy and were in first complete remission. Conditioning before the NK cell transplantation included 60 mg/kg cyclophosphamide for one day and fludarabine 25 mg/m^2 for 5 days. The patients also received six doses of IL-2 given subcutaneously every other day to expand and activate the donor NK cells *in vivo*.

A parental donor with the highest number of KIR mismatches based on our receptor-

Patient characteristics		
Age	Median Range	2.5 years 0.2–21 years
Gender	Male Female	5 5
WBC at diagnosis (10^9/L)	Median Range	62 4–488
FAB classification	M1 M4Eo M5 M7	1 4 3 2
Karyotype	Normal t(1;22) t(9;11) t(11;19) Inv(16) t(16;16) +21	1 2 1 1 1 3 1

Table 1: Patient characteristics.

Recipient HLA ligand	Donor KIR mismatch loci	N
Bw4/Asn80	2DL1	3
Bw4/Lys80	2DL2/3	1
Bw6/Asn80/Lys80	3DL1	1
Bw6/Asn80	2DL1, 3DL1	3
Bw6/Lys80	2DL2/3, 3DL1	1

Table 2: Donor-recipient KIR mismatch based on receptor-ligand model[6].

ligand model was selected (table 2). A single apheresis was performed to obtain peripheral blood mononuclear cells. Donor cells were then selected for NK cells using the CliniMACS® System by a two-step procedure, i.e., $CD3^+$ cell depletion followed by $CD56^+$ cell enrichment.[10] The content of the NK cell grafts is summarized in table 3. Nine of the ten products did not contain any measurable T cells, and one product contained only 10^3 T cells per kg of recipient body weight. Thus, the dose of T cells was below the threshold of 10^5 T cells per kg that is considered to be associated with graft-versus-host disease (GVHD) in our institution. The number of B cells in the graft was also low, therefore limiting the risk of post-transplant lymphoproliferative disease and passenger lymphocyte syndrome

Results

The conditioning, IL-2, and NK cell transplantation were well tolerated.[9] The average hospital stay was 2 days. None of the patients had acute or chronic GVHD.

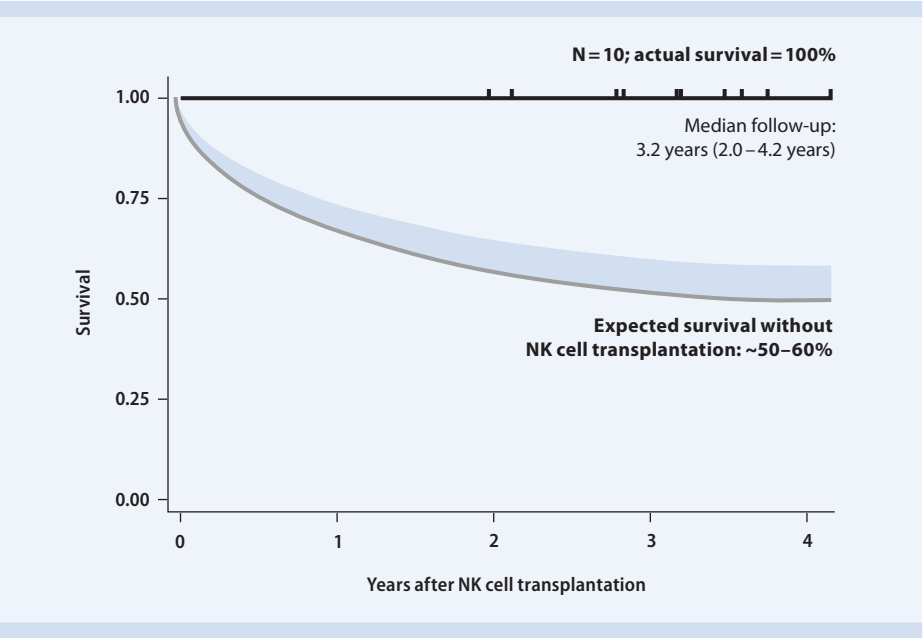


Figure 1: Event-free survival of patients and expected survival.

Cell type	Cell number	
NK cells ($\times 10^6$/kg)	Median Range	29.2 5.2 – 80.9
T cells ($\times 10^6$/kg)	Median Range	< 0.001* < 0.001 – 0.001
B cells ($\times 10^6$/kg)	Median Range	0.097 < 0.001 – 1.70

Table 3: NK cell graft content. *The T cell content in nine of the ten products was below the threshold of detection by flow cytometry analysis (i.e. < 10^3/kg).

With a median follow up of 3.2 years, all patients remain well with no evidence of leukemia >2 years after NK cell transplantation (fig. 1).

Correlative laboratory studies revealed that all patients showed transient donor NK cell engraftment for a median of 10 days (range, 2 –189 days). *In vitro* NK cell cytotoxicity against K562 cells reached normal levels in samples from all patients by day 7 after NK cell transplantation. Importantly, there was a significant expansion of KIR mismatched cells in the blood, from a median of only 210/mL on day 2 to a median of 5,800/mL on day 14.

Discussion

Allogeneic hematopoietic cell transplantation (HCT) is an established treatment for childhood AML.[1] As chemotherapy becomes more successful, most patients with low-risk features can be cured without HCT. However, ~20% of them will subsequently have a leukemia relapse. In intermediate-risk patients, survival probabilities are higher with HCT; however, transplantation is associated with a 16% transplant-related mortality rate, 26% relapse rate, and many acute and late complications.[2,3,11] HCT also leads to considerable financial costs. Furthermore, most of the patients do not have a sibling donor. Taken together, a novel therapy is needed to overcome these limitations.[12]

NK cells can now be isolated easily using an immunomagnetic method resulting in a product with high NK cell purity and little contamination by T and B cells.[10] The two-step procedure can be completed within one day. In pre-clinical models, these purified NK cells show significant activities against leukemia and solid tumors.[13] Therefore, we hypothesized that these NK cells may be useful in the prevention of leukemia relapse after completion of chemotherapy.

As NK cells can be easily obtained from family members by large-volume apheresis, no search for HLA-matched donors is required. Potential family donors can be

screened for optimal NK cell alloreactivity by comprehensive genotyping and phenotyping of donor NK cells.[14,15]

The donor testing can also be accomplished within one day using modern laboratory techniques.[14] Taken together, with the relatively low cost and patient safety profile, NK cell transplantation can now be easily performed even in low-income countries.

Conclusion

NK cell transplantation is safe, feasible, and less costly than sibling donor HCT.[16] If its efficacy is proven in future controlled clinical trials, it may offer a new treatment approach as consolidation therapy in children with low- or intermediate-risk AML.

References

1. Oliansky, D.M. *et al.* (2007) Biol. Blood Marrow Transplant. 13: 1–25.
2. Leung, W. *et al.* (2000) J. Clin. Oncol. 18: 3273–3279.
3. Leung, W. *et al.* (2007) Medicine (Baltimore) 86: 215–224.
4. Imai, K. *et al.* (2000) Lancet 356: 1795–1799.
5. Leung, W. *et al.* (2010) Cancer Epidemiol. 34: 303–308.
6. Leung, W. *et al.* (2004) J. Immunol. 172: 644–650.
7. Bari, R. *et al.* (2013) J. Clin. Oncol. 31: 3782–3790.
8. Bari, R. *et al.* (2009) Blood 114: 5182–5190.
9. Rubnitz, J.E. *et al.* (2010) J. Clin. Oncol. 28: 955–959.
10. Iyengar, R. *et al.* (2003) Cytotherapy 5: 479–484.
11. Horan, J.T. *et al.* (2008) J. Clin. Oncol. 26: 5797–5801.
12. Leung, W. (2009) Semin. Hematol. 46: 89–99.
13. Leung, W. *et al.* (2005) Cancer Immunol. Immunother. 54: 389–394.
14. Bari, R. *et al.* (2011) Clin. Immunol. 138: 274–281.
15. Leung, W. (2005) J. Immunol. 174: 6540–6545.
16. Leung, W. (2014) Clin. Cancer Res. 20: 3390–3400.

Resetting the immune system in severe autoimmune diseases with immunoablation followed by autologous stem cell transplantation using enriched CD34+ cells is associated with long-term remissions – a 15-year single-center experience



Tobias Alexander[1,2] *, Falk Hiepe[1,2], Renate Arnold[3], and Andreas Radbruch[4]

[1] Department of Rheumatology and Clinical Immunology, Charité – University Medicine Berlin, Berlin, Germany, [2] German Rheumatism Research Center Berlin, a Leibniz Institute, Autoimmunology Group, Berlin, Germany, [3] Department of Hematology, Oncology, and Tumor Immunology, Charité – University Medicine Berlin, Berlin, Germany, [4] German Rheumatism Research Center Berlin, a Leibniz Institute, Cell Biology Group, Berlin, Germany, * not shown

MACS Technology in chronic inflammation, autoimmune diseases, and allergy

Andreas Radbruch

When Stefan Miltenyi in our small lab at Cologne University developed MACS Technology for magnetic cell sorting in the late 1980´s, it soon became clear that this technology had an enormous potential both for scientific discovery, but also for clinical application. It was fast, efficient, safe, and gentle to cells. Today, my research group depends on MACS Technology to analyze the cellular basis of immunological memory. It is an indispensable tool for adoptive transfers in mouse immunology and for the isolation of defined cells for their analysis *ex vivo* in human immunology. But above all, Miltenyi Biotec has developed MACS Technology into a system to create defined cell populations for clinical therapy. When I was appointed to the German Rheumatism Research Center (DRFZ) in Berlin in 1997, the challenge was to develop therapeutic strategies for the cure of chronic inflammatory diseases. We aimed for an extinction of the pathogenic immunological memory for inflammation and a restoration of immunological tolerance in the patients. In 1998, together with Stefan Miltenyi, a scientific consortium of the DRFZ and clinical experts from the Charité – University Medicine developed a therapeutic concept of complete immunoablation followed by transplantation of purified autologous hematopoietic stem cells, to "reset" the immune system in patients with severe autoimmune diseases, using the CliniMACS System as an essential tool for stem cell purification. This experimental therapy has induced therapy-free remission for up to 15 years by now, in many patients who had been refractory to conventional therapy before. And as proof of principle this therapy has identified new targets for the treatment of chronic inflammation, autoimmune diseases, and allergies. MACS Technology has played and still is playing an essential role in this breakthrough discovery.

Introduction

Chronic immunosuppression and novel biologic therapies can suppress or attenuate the inflammatory process in autoimmune disease (ADs) as long as they are applied, but cannot switch off the underlying mechanisms to induce therapy-free remission, i.e., cure. Although effective in most cases, immunosuppression is associated with reduction in quality of life, cumulative toxicity, increased risk of cardiovascular disease, and represents a considerable socio-economic challenge. For patients with major organ involvement and therapy-resistant disease, high-dose immunosuppression followed by autologous hematopoietic stem cell transplantation (ASCT) has been used since 1995[1] worldwide and was shown to induce treatment-free remissions in several ADs.[2–6] Based on experimental data from animal models[7,8], immunoablation and ASCT for ADs is applied with the goal to eradicate the autoreactive immunologic memory and to regenerate a naive and self-tolerant immune system from hematopoietic precursors. Our

previous data and mechanistic studies from other groups have meanwhile provided the "proof of concept" that a chronic autoreactive immune system can indeed be "reset" into a naive and self-tolerant immune system. These data include the regeneration of naive B cells[2,8], thymic reactivation[2,10–12], re-emergence of a polyclonal TCR repertoire[2,10,12], and restoration of FOXP3+ regulatory T cell (Treg) levels[13,14]. Here, we describe the clinical and serologic responses and long-term immune reconstitution in 20 patients with severe ADs for up to 15 years after receiving immunoablation and ASCT, and show that "resetting" the immune system is associated with durable long-term clinical remissions in ADs despite discontinuation of immunosuppressive therapies. Remissions were associated with i) the depletion of the autoreactive immunologic memory, reflected by the disappearance of pathogenic and protective antibodies, and ii) a profound reconfiguration of the adaptive immune system with a stable reactivation of the thymus and re-emergence of thymic naive FOXP3+ Tregs, in other words, a "reset of the immunological clock".

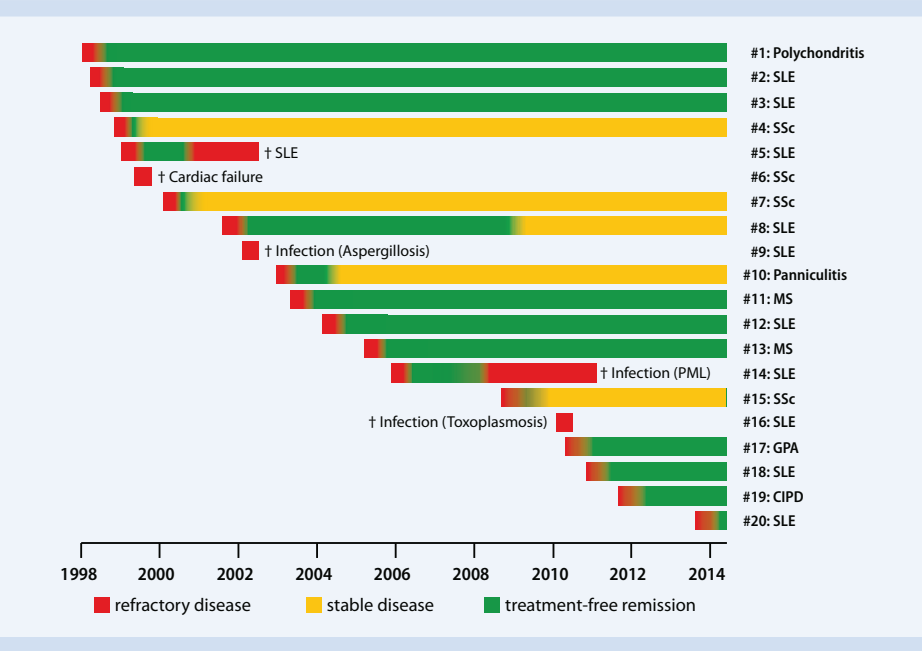


Figure 1: Immune reset is associated with long-term clinical remissions in autoimmune diseases. To illustrate the clinical outcome of patients with autoimmune diseases after immune reset, patients were classified as having treatment-free remission (green), stable disease (yellow), or refractory disease (red). SLE: systemic lupus erythematosus; SSc: systemic sclerosis; MS: multiple sclerosis; GPA: granulomatosis with polyangiitis; CIPD: chronic inflammatory demyelinating polyneuropathy; PML: progressive multifocal leukencephalopathy.

Material and methods

Study design and clinical trial protocol

In this prospective single-center study, patients with ADs were included who received immunoablation and ASCT as part of a phase I/II clinical trial (registered at www.clinicaltrials.gov as NCT00742300) after failure of remission despite two different immunosuppressive therapies. The clinical trial was approved by the responsible ethics committee and was conducted in accordance with the Declaration of Helsinki. A detailed description of the trial protocol and the included patients has been published previously.[7,8] Peripheral blood stem cells were collected by leukapheresis after infusion of 2.0 g/m² cyclophosphamide followed by daily granulocyte colony-stimulating factor (10 µg/kg; Amgen). The graft was enriched for CD34+ cells using the CliniMACS® Plus Instrument (Miltenyi Biotec). Immunoablation was achieved by 200 mg/kg of cyclophosphamide and 90 mg/kg rabbit ATG (Neovii Biotech, formerly Fresenius).

Serological analysis

Anti-nuclear antibodies (ANA) were assessed by indirect immunofluorescence on HEp-2 cells. Anti-double-stranded DNA (anti-dsDNA) antibodies and protective antibodies (vaccine titers) for tetanus toxoid were detected by commercial ELISA.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinized blood by Ficoll™ Hypaque™ density gradient centrifugation (Pharmacia Biotec). The phenotypes of the cells were determined by flow cytometry using the following antibodies: anti-CD3 (UCHT1, Biolegend), anti-CD4 (TT1, BD™ Biosciences), anti-CD31 (AC128, Miltenyi Biotec), and anti-CD45RA (L48, BD Biosciences). FOXP3 staining was performed after fixation and permeabilization (FOXP3 buffer, eBioscience) using anti-FOXP3 (PCH101, eBioscience). Cells were washed before acquisition for flow cytometry (FACSCalibur™ and FACSCanto™ Flow Cytometers, BD Biosciences, with FlowJo Software, TreeStar). Quantification of peripheral blood lymphocyte subsets was performed with the TruCount™ System (BD Biosciences). Data were analyzed using FlowJo Software v7.6.5.

Statistical analysis

A non-parametric matched paired test (Wilcoxon) was used to compare (per patient and immune parameter) pre- and post-transplant data using GraphPad Prism® 5 software (Graph Pad Software Inc.). Based on distributional assumptions, the Mann-Whitney *U* test was used to compare data from patients treated by ASCT with those from healthy donors.

Results

Immune reset is associated with clinical long-term responses in autoimmune diseases

From February 1998 until August 2013, 20 patients with different autoimmune diseases received an ASCT with enriched CD34+ cells, at the Charité – University Medicine Berlin (fig. 1). Major indications for immune reset were systemic lupus erythematosus (SLE, n = 10) and systemic sclerosis (SSc, n = 4), followed by multiple sclerosis (MS, n = 2), polychondritis (n = 1), panniculitis (n = 1), granulomatosis with polyangiitis (GPA, n = 1), and chronic inflammatory demyelinating polyneuropathy (CIPD, n = 1). All patients had persistently active disease despite standard

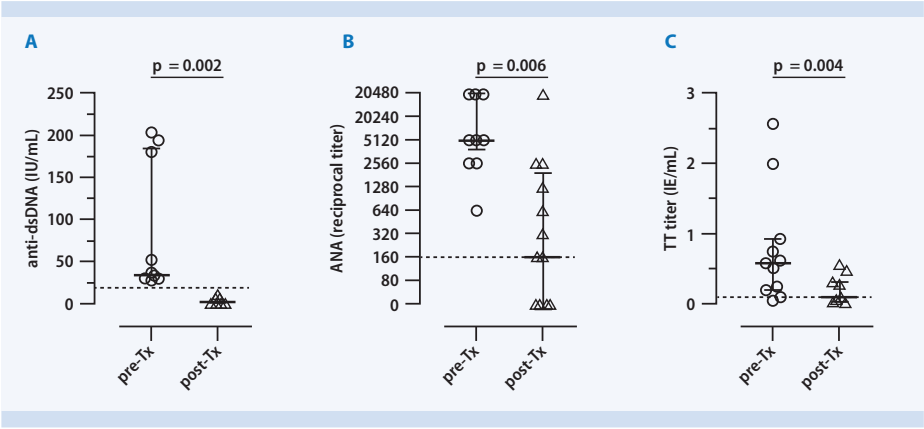


Figure 2: Significant reduction of serum (auto)antibody titers after immune reset. (A) Serum titer of anti-dsDNA antibodies at baseline before ASCT (pre-Tx) and at 6 months after transplantation in ten SLE patients. (B) Serum titer of antinuclear antibodies (ANA) before ASCT and 6 months after ASCT (post-Tx) in all patients with connective tissue diseases (SLE, n=10 and SSc, n=4). (C) Serum antibody titers against tetanus toxoid (TT).

immunosuppression before enrollment. With a median follow-up of 12 years after immune reset (range, 18 months to 16 years), 15 of 20 patients (75%) achieved a progression-free survival, defined as survival without major organ failure. From these patients 10 of 20 (50%) had long-term clinical remission for up to 15 years despite discontinuation of immunosuppressive treatment, while 5 of 20 (25%) had stabilization of their underlying disease under reintroduction of maintenance therapies. Transplant-related mortality (TRM), defined as death within the first 100 days after transplantation, was 12% with 3 of 20 patients dying from infection (n = 2, aspergillosis and toxoplasmosis) or cardiac failure (n = 1). Relapse of the underlying autoimmune disease occurred in three SLE patients (at 18, 36, and 80 months, respectively), two of whom died later from uncontrolled disease and infection.

Clinical remissions after immune reset are associated with the depletion of the autoreactive immunologic memory

All patients with connective tissue diseases (SLE and SSc) had serum anti-nuclear antibodies (ANAs), and SLE patients had persistently elevated anti-dsDNA antibodies before enrollment, despite intensive immunosuppression. After immune reset, anti-dsDNA antibodies completely normalized in all SLE patients (fig. 2A) and ANA significantly declined from a median titer of 1:5120 at baseline to 1:160 six months after transplantation in all patients with connective tissue diseases (p = 0.006, fig. 2B). ANA titers even became negative or decreased to titers of 1:160 or below, which is regarded as clinically irrelevant, in 7 of 10 SLE patients. This is remarkable as these ANA titers are usually not affected by conventional immunosuppressive therapies. Not only autoantibodies, but also protective antibodies (vaccine titers) against tetanus toxoid significantly declined in serum after immune reset (p = 0.004, fig. 2C). This drastic ablation of humoral memory suggests that the ATG used for immunoablation directly targets the plasma cells (PCs) secreting these serum antibodies. In line with this hypothesis, we were able to show that PCs disappeared from bone marrow one month after immunoablation in one case, as described earlier.[2] Notably, insufficient reduction of ANAs in SLE patients after immune reset was associated with a higher risk for flare induction. From 3 of 10 SLE patients with incomplete reduction of ANA titers, two patients later developed a disease flare, while all but one patient with ANA disappearance were in long-term remission.[15]

Stable thymic reactivation contributes to the regeneration of FOXP3$^+$ regulatory T cells

A stable thymic reactivation with replenishment of thymic naive T cells is a prerequisite to re-establish central tolerance after immune reset.[2,11] To assess the number of recent thymic emigrants (RTEs), CD31-expressing CD45RA$^+$CD4$^+$ T cells were investigated by flow cytometry before and after immune reset. At baseline, numbers of RTEs were significantly decreased in enrolled patients compared to age-matched healthy donors (median: 45.0/µL vs. 164.0/µL, p = 0.001), reflecting the disturbed T cell homeostasis in SLE patients and/or effects of immunosuppression (fig. 3A). Recovery of RTEs to numbers comparable to healthy donors was completed between 12 and 24 months after immune reset, reaching on average 3.6 to 5.1 times the baseline levels. Remarkably, the number of RTEs continued to increase in responding patients, and it was not until 36 months after ASCT, when RTEs reached a plateau, which was more than twice as high as the level observed in age-matched healthy donors (median: 392.0/µL vs. 164.0/µL, p = 0.006). The level returned to the range of those from healthy donors, but not until 8 years after transplantation.

In addition, we investigated the phenotype of recurring FOXP3$^+$ Tregs after immune reset by assessing their surface expression of CD45RA and CD31 by flow cytometry. In healthy donors, Treg expression levels of CD45RA and CD31 decreased from a median of 62.4% in cord blood to 3.1% at the age of 80 years, reflecting a decline in thymic output during aging (fig. 3B). When recurring Tregs after immune reset in SLE patients were analyzed, their surface expression of CD45RA and CD31 was significantly higher compared to age-matched healthy donors even at later times after transplantation, indicating that the majority of FOXP3$^+$ Tregs were newly generated from the thymus. Notably, SLE patients who relapsed had lower surface expression levels of CD45RA and CD31 on Tregs compared to patients with long-term remission, suggesting that a stable replenishment with thymic naive Tregs is a prerequisite for durable remissions. We also investigated Treg expression of Helios, a marker recently proposed to differentiate thymic-derived from peripherally induced Tregs[16], by flow cytometry. Surprisingly, Helios, in contrast to CD31, was stably expressed in Tregs from healthy donors at an average of approximately 70% during aging. Moreover, Helios was expressed at similar levels in Tregs from patients after immune reset and healthy donors.[17] This suggests that Helios is not a suitable marker to identify recently generated thymic-derived Tregs.

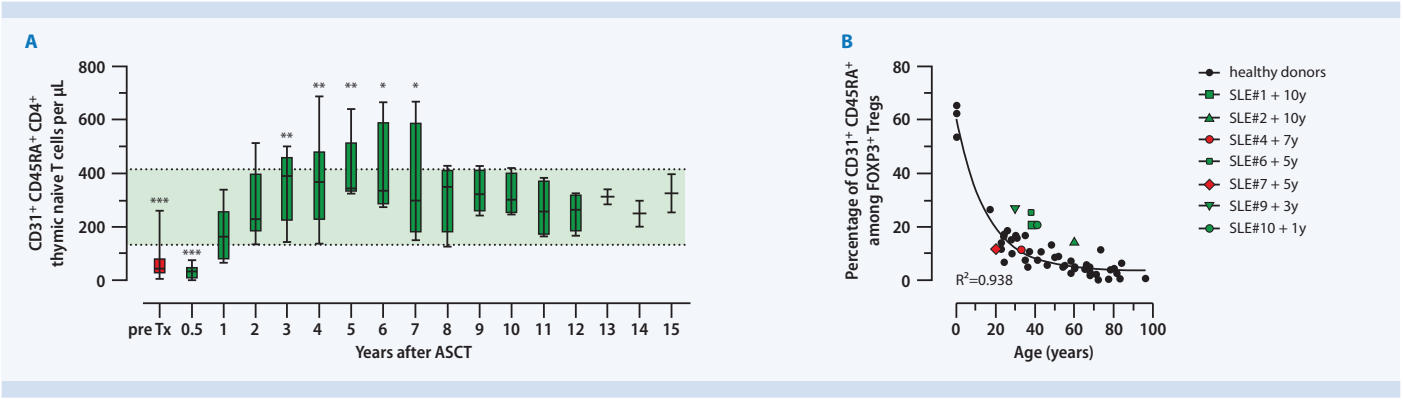


Figure 3: Stable thymic reactivation after immune reset contributes to regeneration of thymic naive FOXP3⁺ regulatory T cells. (A) Absolute counts of thymic naive CD31⁺CD45RA⁺CD4⁺ T cells in 20 patients before ASCT (pre-Tx) and during the time course after ASCT. Normal ranges were established based on analysis of 28 age-matched healthy donors. Depicted are 5th and 95th percentiles (light green area). A Mann-Whitney *U* test was used to compare data from patients before and after immune reset with those from healthy donors. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$). (B) Frequencies of CD31 and CD45RA-expressing FOXP3⁺ Tregs in healthy donors over age (black dots) and SLE patients at depicted time points after immune reset. Data from patients with long-term remission are depicted in green symbols while those after disease relapse are depicted in red symbols.

Conclusion

Immune reset with immunoablation followed by ASCT has emerged as a promising salvage therapy for severely affected AD patients, but data on the long-term efficacy of this treatment approach are limited. Here, we describe the clinical and serologic responses and long-term immune reconstitution data from 20 patients with severe ADs prospectively followed up for a maximum of 15 years after receiving immunoablation and ASCT. We show that this treatment is associated with durable long-term clinical remissions despite discontinuation of chronic immunosuppression. Our detailed analysis of the long-term reconstitution of the patients' immune systems with respect to the recurrence of T cell subsets and the course of serologic changes over time demonstrated a successful depletion of autoreactive immunological memory and the regeneration of a tolerant immune system from hematopoietic stem cells, in other words, a "reset of the immunological clock".

Depletion of the autoreactive immunologic memory is best reflected by a drastic reduction or even disappearance of both protective (vaccine-specific) antibodies and pathogenic autoantibodies, such as anti-nuclear antibodies. These antibodies are secreted by long-lived PCs that reside in dedicated survival niches in the bone marrow or inflamed tissues.[18] Long-lived PCs are resistant to conventional immunosuppressive and/or B cell–depleting therapies and may thus contribute to the maintenance of chronic autoimmunity and/or flare induction in antibody-mediated autoimmune diseases, such as SLE, by the persistent secretion of these autoantibodies.[18] Depletion of autoreactive PCs during immunoablation was achieved by polyclonal rabbit ATG that directly targets PCs and B cells via complement-mediated lysis and apoptosis.[20] In line with this notion, we were able to stain PCs *ex vivo* with the polyclonal rabbit-ATG used for immunoablation; moreover, PCs disappeared from the bone marrow one month after immunoablation in one case, as described earlier.[2] Notably, insufficient depletion of the autoreactive immunologic memory was associated with a higher risk to flare in SLE patients after immune reset.[15] But not only sufficient *in vivo* depletion with ATG is essential to induce durable remissions, also *ex vivo* manipulation of the stem cell graft with CD34⁺ cell selection had a significant effect on the long-term outcome of patients. Retrospective data analyzing the clinical responses of 28 SLE patients reported to the EBMT between 2001 and 2008 after immune reset, including our patients, recently showed that the risk of flare induction after ASCT was significantly lower in SLE patients who received a graft of enriched CD34⁺ stem cells compared with those without graft manipulation (11% vs. 68%, p = 0.001).[4] These data indicate that a successful eradication of the autoreactive memory by both *in vivo* and *ex vivo* depletion is a prerequisite to achieve durable remissions after immune reset in ADs.

Regeneration of the immune system after ASCT involved a stable reactivation of the thymus, characterized by a continuous re-emergence of RTEs reaching significantly higher absolute numbers for up to 7 years following ASCT compared to age-matched controls. This is of particular relevance for the replenishment with thymic naive FOXP3⁺ Tregs that are essential to re-induce self-tolerance after immune reset. In line with this notion, repopulating Tregs after immune reset in responding SLE patients predominantly displayed a thymic naive CD45RA⁺CD31⁺ phenotype. Stable thymic reactivation seems to be a prerequisite for long-term remissions as patients with delayed thymic reactivity after transplantation and lower levels of natural Tregs were at higher risk to develop disease flares.[15]

In conclusion, these data confirm our assumption that the reprogramming of an autoreactive immune system into a juvenile and self-tolerant immune system is feasible and associated with long-term remissions in ADs, which are caused by unknown triggers based on a polygenic background. Long-term remissions rely on a sufficient eradication of the autoreactive immunologic memory, achieved by *in vivo* depletion regimens such as ATG, combined with *ex vivo* graft purging using CD34⁺ cell selection, as well as a stable thymic reactivation with re-emergence of thymic naive Tregs. Our findings propose that chronic autoimmunity is not an end point depending on continuous treatment with specific anti-inflammatory agents, but may be cured by combining specific targeting of autoreactive

memory and effector cells with a reactivation of thymic activity. A future challenge is to make this therapeutic approach attractive for a larger number of patients. For this purpose the rate of severe infections has to be reduced by accelerating the reconstitution of the protective immunological memory. Therefore, our research is focused on the selective depletion of the pathogenic cells sparing the protective memory to a large extent or the early addition of protective immune cells. This may be achieved by either using a more selective graft purging, e.g., depletion of T cell receptor alpha/beta and $CD19^+$ cells from apheresis products with the CliniMACS Device,[21] or an adoptive transfer of microbe- or virus-specific memory T and/or B cells.

References

1. Gratwohl, A. *et al.* (1997) Bone Marrow Transplant. 19: 407–419.
2. Alexander, T. *et al.* (2009) Blood 113: 214–223.
3. Farge, D. *et al.* (2010) Haematologica 95: 284–292.
4. Alchi, B. *et al.* (2013) Lupus 22: 245–253.
5. van Laar, J.M. *et al.* (2014) JAMA 311: 2490–2498.
6. Burt, R.K. *et al.* (2009) Lancet Neurol. 8: 244–253.
7. van Bekkum, D.W. (1993) Bone Marrow Transplant. 11: 183–187.
8. Ikehara, S. (2008) Autoimmunity 41: 563–569.
9. Szodoray, P. *et al.* (2012) Scand. J. Rheumatol. 41: 110–115.
10. Muraro, P.A. *et al.* (2005) J. Exp. Med. 201: 805–816.
11. Thiel, A. *et al.* (2008) Acta Haematol. 119: 22–27.
12. Farge, D. *et al.* (2005) Arthritis Rheum. 52: 1555–1563.
13. de Kleer, I. *et al.* (2006) Blood 107: 1696–1702.
14. Abrahamsson, S.V. *et al.* (2013) Brain 136: 2888–2903.
15. Alexander, T. *et al.* (2013) Bone Marrow Transplant. 48 (suppl. 2): S336.
16. Thornton, A.M. *et al.* (2010) J. Immunol. 184: 3433–3441.
17. Alexander, T. *et al.* (2013) Ann. Rheum. Dis. 72: 1549–1558.
18. Radbruch, A. *et al.* (2006) Nat. Rev. Immunol. 6: 741–750.
19. Hiepe, F. *et al.* (2011) Nat. Rev. Rheumatol. 7: 170–178.
20. Zand, M.S. *et al.* (2005) Transplantation 79: 1507–1515.
21. Schumm, M. *et al.* (2013) Cytotherapy 15: 1253–1258.

Sorting for cell therapy

Xianmin Zeng[1] and Mahendra Rao[2]
[1]Buck Institute for Research on Aging, Novato, CA, USA
[2]NIH Center of Regenerative Medicine, Bethesda, MD, USA

Cell-based therapy for treating neurological disorders is in its infancy and currently only four to five companies are using neural cells for treating neural diseases. There are several reasons for this, including the lack of sufficient cells, our inability to direct differentiation to an appropriate phenotype, and our difficulty in delivering the cells in sufficient numbers to the right region of the brain. Several technological breakthroughs have helped resolve some of these issues. These include the development of pluripotent cells, the ability to direct their differentiation to an appropriate phenotype, and the ability to identify cell surface markers present at the right stage of development to allow one to transplant cells. In this article we review potential markers and technologies for selecting cells.

Introduction

Neural development follows a characteristic program that involves a progressive restriction in developmental potential and several stable intermediates that can be distinguished from each other. A generalized model of such differentiation is provided in figure 1. Such differentiation post fertilization can be replicated *in vitro* from pluripotent stem cells (PSC). Embryonic stem cells (ESC) or induced PSC (iPSC) can be primed to the epiblast stage and then directed to make neuroectoderm via an *in vitro* model of gastrulation termed embryoid body formation.

A subset of cells in the embryoid body are fated to make the central nervous system, and these cells can be identified by their ability to make neural rosettes upon adherent culture of the embryoid bodies. These neural rosettes can be manually picked and propagated in defined medium allowing one to obtain a relatively pure homogenous population of cells.

An important finding from gene array analysis of these rosette-derived neural stem cells (NSC) was that they are positionally naive and as such are capable of making neurons from any part of the rostrocaudal axis. Positional specification of these cells can be achieved by the use of growth factors such as FGFs, Shh, BMPs, and retinoic acid in specific combinations.

The same NSC can be directed to make oligodendrocytes and astrocytes as well, and what has become clear is that additional, more restricted stages of dividing precursor cells are present, which can be used as intermediate stages for cryopreservation.

Our ability to control the process of differentiation *in vitro,* while impressive, is still limited in terms of obtaining near homogenous populations of differentiated cells. In general, unless a purification strategy is used, one obtains an enriched population by directed differentiation that is contaminated with precursors, progenitors, or unwanted differentiated cells.

The same basic principle is largely true for the peripheral nervous system (PNS) as well. The neural crest stem cells diverge from the CNS-NSC early in development and they generate sympathoadrenal and sensory precursors, and the PNS glial cells (Schwann cells). The factors that direct differentiation appear to be the same as in the CNS, although the timing of application and the type of differentiation they direct is different. For example, BMPs promote neurogenesis in the PNS while they promote gliogenesis in the CNS.

The ability to obtain large populations of enriched cells has led to several efforts to utilize them for therapy, and several trials are underway or planned with NSC and glial progenitors and more recently with dopaminergic neurons. What has become clear from these early attempts is that it is important to be able to reduce variability in manufacture and to obtain a sufficiently pure population. To a certain degree, cell purification limits the numbers of cells that can be used for effective therapy. While for NSC this limitation was eliminated by cell culture methodology with reasonable success, this has proven more difficult for other cell types and in particular subtypes of neurons.

Several strategies have been attempted using flow sorting, bead-based selection with cell surface markers, and genetic engineering to engineer a suicide gene or a drug resistance gene for positive and negative selection. Each of these techniques has its own merits.

In the next section we will briefly discuss our strategy and rationale for selection.

Choosing bead-based selection over alternatives

Our initial goal was to obtain purified cell populations for the treatment of Parkinson's disease and oligodendrocyte precursors for the treatment of demyelinating disorders. Our first decision was which of the alternative selection strategies we could use. We found that media selection strategies were not reliable, and directed differentiation – while good for enrichment – did not provide consistency from lot to lot. We discarded gene engineering as a first choice because getting efficient targeting on a consistent basis was difficult. Moreover, it introduced additional regulatory hurdles and increased the chance of developing antibodies to foreign proteins that were incorporated for selection strategies. Developing inducible, excisable constructs also had issues in the complexity of vector design and silencing.

Antibody-based selection on the other hand appeared attractive as there was a rich literature on potential markers from the developmental biology experiments carried out in multiple species. There were several antibodies that were already available, and techniques for humanizing antibodies or developing novel antibodies had been optimized based on

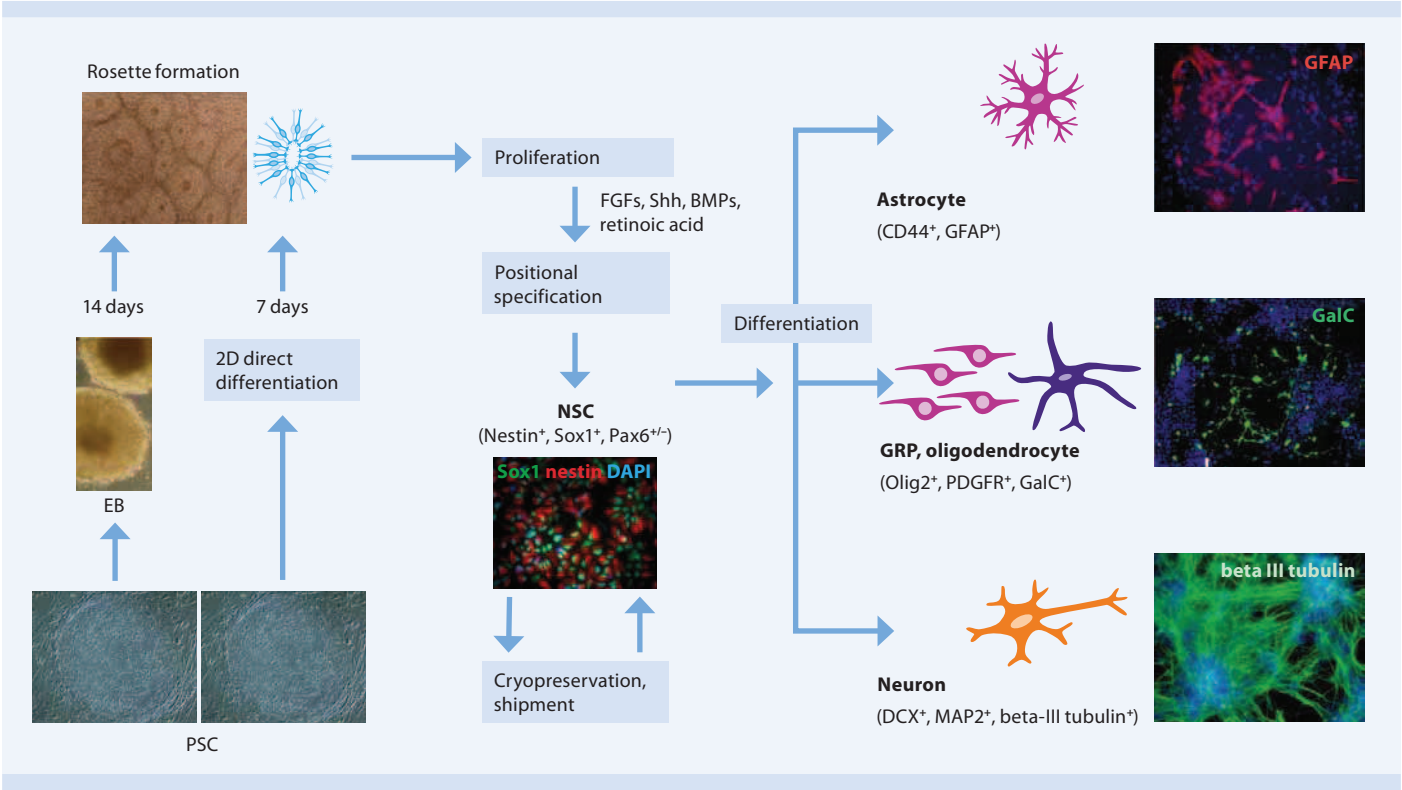


Figure 1: Model for the differentiation of pluripotent stem cells (PSC) into astrocytes, oligodendrocytes, and neurons.
EB: embryoid body; NSC: neural stem cell; GRP: glial-restricted precursor.

work that had been done during development of antibodies for immunotherapy. Effective methods for conjugating antibodies to beads were also available, as were flow cytometry–based methods of live cell sorting with the development of automated laser-based selection strategies.

Two major reasons made the selection between bead-based technologies and flow sorting–based technologies easier for us. Flow sorting devices have been difficult to build as closed systems that would pass regulatory approval while cGMP-compliant systems for bead-based sorting were readily available. The second was speed of sorting and scalability along with the ability to couple positive and negative sorting procedures with multiple columns. A third reason, while not true for all cell types, was important for neuronal precursors. These cells are a lot more sensitive to sorting, and bead-based sorting appeared gentle enough that these cells could be sorted relatively efficiently. Although these reasons were sufficient for us to choose bead-based sorting, given the state of technology today it is important to emphasize that bead-based sorting has its disadvantages. These include issues such as purity of the final product, which in our hands is never as good as with flow sorting, the issue of antibody leaching, the cost of developing a cGMP-compliant antibody, and the retention/ingestion of beads.

Potential markers that can be used for antibody-based selection in the CNS

We and others have tested a variety of cell surface markers that can be used for cell selection and these are summarized in figure 2. These include markers for neurons, astrocytes, and oligodendrocyte precursors. The antibodies are widely available and cGMP-compliant versions of the antibody are available as well. As can be seen from the list, it is possible to select at different stages in development, and with use of positive and negative selection markers one can efficiently obtain a consistent end product from a manufacturing run.

We were intrigued to note that Miltenyi Biotec offers many of these antibodies and a sorting kit with non-cGMP compliant antibodies for testing at a reasonable cost. Miltenyi Biotec also offers an upgrade/scalable path that allows one to rapidly test on small lots and graduate to bulk sorting on an automated system with a fairly reasonable certainty that the process will work. A representative figure for purification of CD271+ neural crest stem cells is shown in figure 3.

In collaboration with Q therapeutics we tested a cGMP-qualified A2B5 subclone for purifying oligodendrocyte precursors, the use of PSA-NCAM for purifying neuronal precursors, and CD44 for purifying astrocyte precursors. This was also successful. We tested the viability of the cells after a freeze/thaw cycle and found that there was a small reduction in viability if cells were frozen soon after sorting but that waiting an additional period of time after sorting (48–72 h) allowed cells to recover and enabled us to bank cells efficiently.

An important learning experience for us was that the enzymes used for dissociating our adherent cell cultures had to be carefully selected and neutralized as if one did not pay careful attention to this detail, one either degraded the epitope on the cells or the antibody present on the beads. In general,

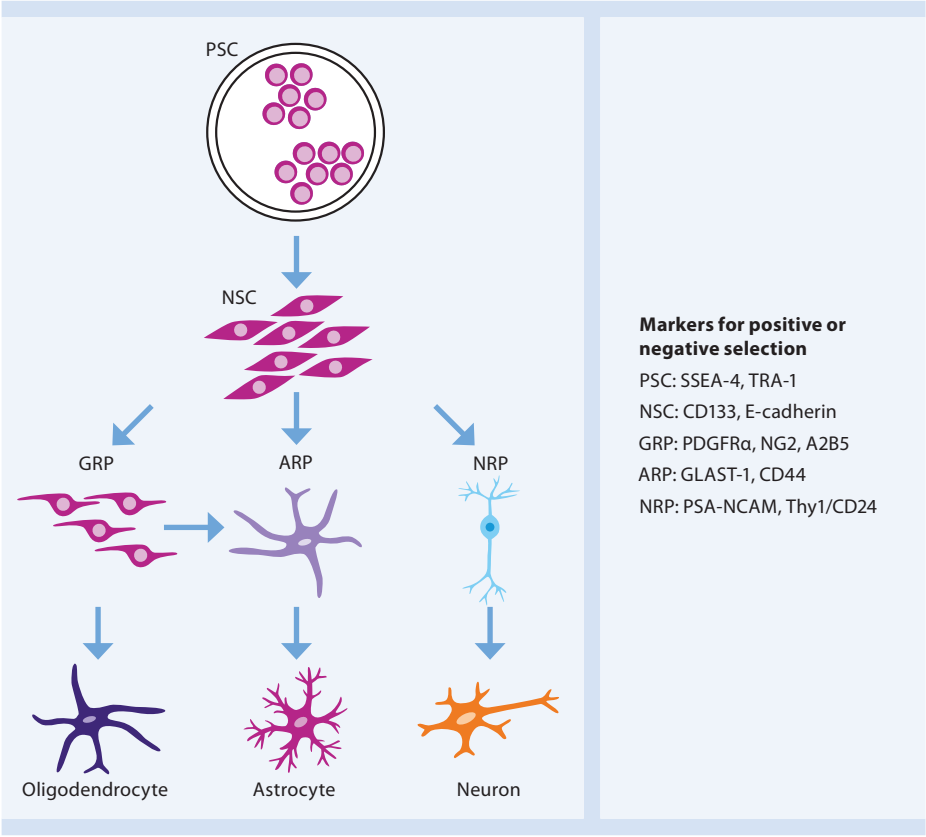


Figure 2: Differentiation of neural stem cells (NSC) into the various lineages, and markers for the isolation of the different stem and precursor cells. PSC: pluripotent stem cell; GRP: glial-restricted precursor; ARP: astrocyte-restricted precursor; NRP: neuronal-restricted precursor

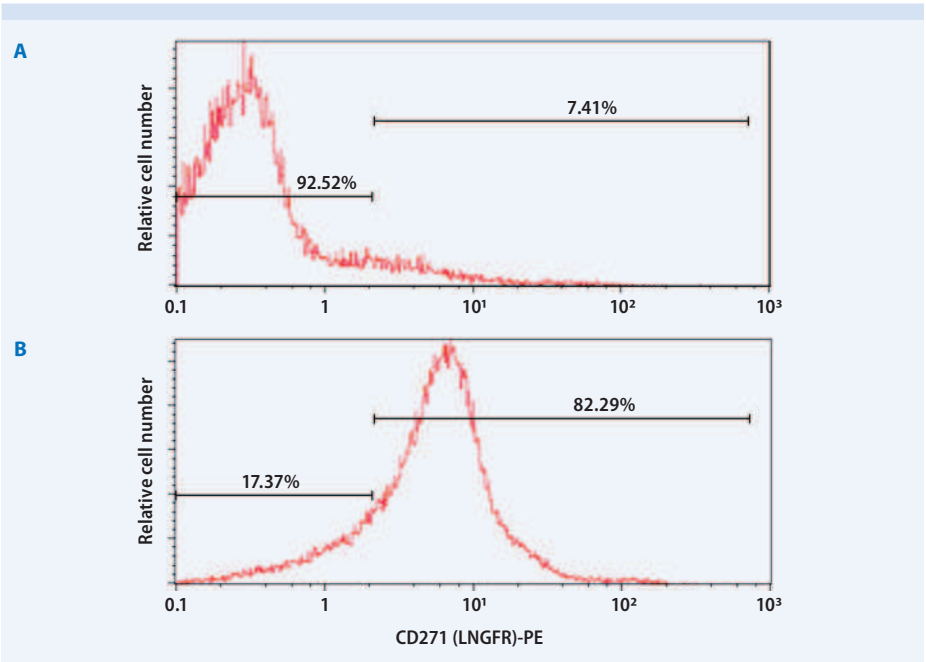


Figure 3: Enrichment of human neural crest stem cells from pluripotent stem cells based on the expression of CD271. Cells were isolated using the CD271 MicroBead Kit and the autoMACS® Pro Separator. Cells before (A) and after separation (B) were stained with CD271 (LNGFR)-PE and analyzed by flow cytometry.

dissociation enzymes that affect the substrate rather than the cell receptor are preferred.

A second important learning was making sure that cells were dissociated into single cells. Having clumps increased the number of contaminating cells, particularly when we were sorting neurons as astrocytes appear to adhere to clusters of neurons.

A third important learning was that sorting is preferably done as close to the final product as possible as a small contamination of dividing cells within the sorted cell population can take over a dish after longer periods of culturing. It was therefore important to measure the degree of contamination (and viability) by immunocytochemistry at the time of cryopreservation.

A fourth important learning was that cryopreservation and thawing affect different cells differently, and if one stores a mixed population of neurons and stem cells or neurons and astrocytes or oligodendrocyte precursors and astrocytes, then one enriches for the more resistant cell. Given that the viability can be as low as 70%, it is possible to have a 90% pure population of neurons stored, and then thaw and find that 30% of the neurons were lost, which increases the proportion of the contaminating astrocytes dramatically.

Conclusion

Overall we have found bead-based sorting to be an effective tool in selecting multiple populations of cells for therapy. It has been relatively easy to test, scale up, and use. The availability of bead-based sorting techniques, the availability of antibodies, and the history of regulatory approvals gives one a feeling that at least some of the inherent risks in any translation have been mitigated.

The CD271 MicroBead Kit and the autoMACS Pro Separator are for research use only and not for therapeutic or diagnostic use.

Engineering CARs: How the idea of redirecting an immune response takes the front seat

Hinrich Abken
Center for Molecular Medicine Cologne, University of Cologne, and Dept. I Internal Medicine, University Hospital Cologne, Cologne, Germany

It is generally believed that the immune system can control cancer in the long term. In the late 1980s the hope to cure cancer by adoptive immune cell therapy was fueled by technical progress made in redirecting cytolytic immune cells specifically towards pre-defined targets. Patient's immune cells are *ex vivo* engineered with a recombinant receptor molecule, the so-called chimeric antigen receptor (CAR), whose extracellular part binds to the target and thereby initiates cell activation through the intracellular part. The implementation of such cancer-specific cell therapy, however, is challenging and involves several *ex vivo* manipulations, which at that time were not fully established and far away from routine clinical practice. It was Stefan Miltenyi and his team who shared in those early phases the vision of a specific immune cell therapy and pioneered the development of reagents and devices, which allow for the production of modified cells in relevant numbers and at the GMP level. Further exploration of the scientific concept and the significant progress in the CAR design during the last decade enabled spectacular success in most recent early phase trials. Although more and more clinical trials are convincing a growing community of scientists and clinicians that adoptive cell therapy with specific effector cells will help to fight cancer, the *ex vivo* engineering and amplification of such modified cells still remain crucial steps in the implementation to clinical practice. We here briefly discuss recent advances in the field.

The promise of adoptive T cell therapy for cancer

Metastatic cancer still remains difficult to treat, despite newly developed drugs with clear palliative benefit for the majority of patients, and treatment frequently fails to control cancer in the long term. Experimental and clinical evidence, however, indicates that the immune system is capable of identifying and destroying cancer cells specifically. Adoptive cell therapy with tumor-infiltrating lymphocytes (TILs) in combination with a non-myeloablative lymphodepletion regime has shown some success in the treatment of chemotherapy-resistant melanoma, even in advanced stages of the disease[1]. TILs isolated from tumor lesions, amplified *ex vivo,* and re-administered to the patient produce a powerful anti-tumor response and induce an acute inflammatory reaction, which attracts a second, antigen-independent wave of immune cell invasion into the same lesion. A number of early-phase trials demonstrate that such adoptive cell therapy can result in long-term benefits even after short-term treatment. The TIL strategy, however, has some limitations in the clinical application to a broad variety of cancer as there is currently only a small range of malignancies from which tumor-reactive TILs can be successfully isolated and amplified.

The CAR strategy: T cells redirected by a composite receptor molecule

As the use of TILs in adoptive cell therapy poses certain limitations, the idea of *ex vivo* modification of patient's peripheral blood T cells with pre-defined specificity has generated growing interest. T cells were engineered with a T cell receptor (TCR) by genetic engraftment with the α and β chain, which provide, in addition to the pre-existing specificity, a new specificity for a pre-defined target. The procedure was substantially simplified by the pioneering work of Zelig Eshhar (Weizmann Institute of Science) who demonstrated that T cells can be redirected by a composite single-chain receptor molecule, a chimeric antigen receptor (CAR). The extracellular part of the CAR is composed of an antibody-derived domain for binding to

the target, i.e., a defined surface molecule. The intracellular part consists of a TCR-derived CD3ζ domain to provide T cell activation upon engagement of target (fig. 1)[2,3]. Such CAR-modified T cells are also known as "T-bodies". The CAR was initially called "immunoreceptor", indicating both antibody and receptor parts of the composite molecule in a single term. CAR binding to cognate antigen on the tumor cell surface results in CAR clustering on the engineered T cell. This in turn results in the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the signaling moiety, thereby initiating a downstream signaling cascade, which finally induces T cell amplification, cytokine secretion, and cytolytic activity of the CAR T cell towards the cognate tumor cell. CAR-expressing T cells can be isolated to high purity by magnetic cell separation (fig. 2).

The CAR strategy provides a number of advantages for clinical use. By utilizing an antibody for cognate antigen binding, target recognition becomes independent of antigen processing and major histocompatibility complex (MHC) presentation. Moreover, CARs provide the possibility to recognize non-classical T cell antigens, including carbohydrates, which are frequently altered in tumor cells[4–6]. The TCR, in contrast, is restricted to the recognition of specific peptides presented by the particular MHC. Antibody-mediated target recognition by CARs, however, does not exclude targeting MHC-presented antigens as exemplarily shown by targeting HLA-A*0201–presented NY-ESO1 peptide[7]. Cytotoxic T lymphocytes (CTLs) engineered with a CAR specific for a tumor-associated antigen were specifically activated, and consequently secreted pro-inflammatory cytokines, induced tumor cell lysis *in vitro,* and eradicated transplanted tumors in a variety of mouse models.

Second-generation CARs: different signals shape the T cell response differently

Despite potent *in vitro* activity of CAR-engineered T cells, first clinical trials showed limited therapeutic efficacy[8–10]. This is attributed to a transient activation and poor persistence of engineered T cells once applied to the patient. To evade anergy and activation-

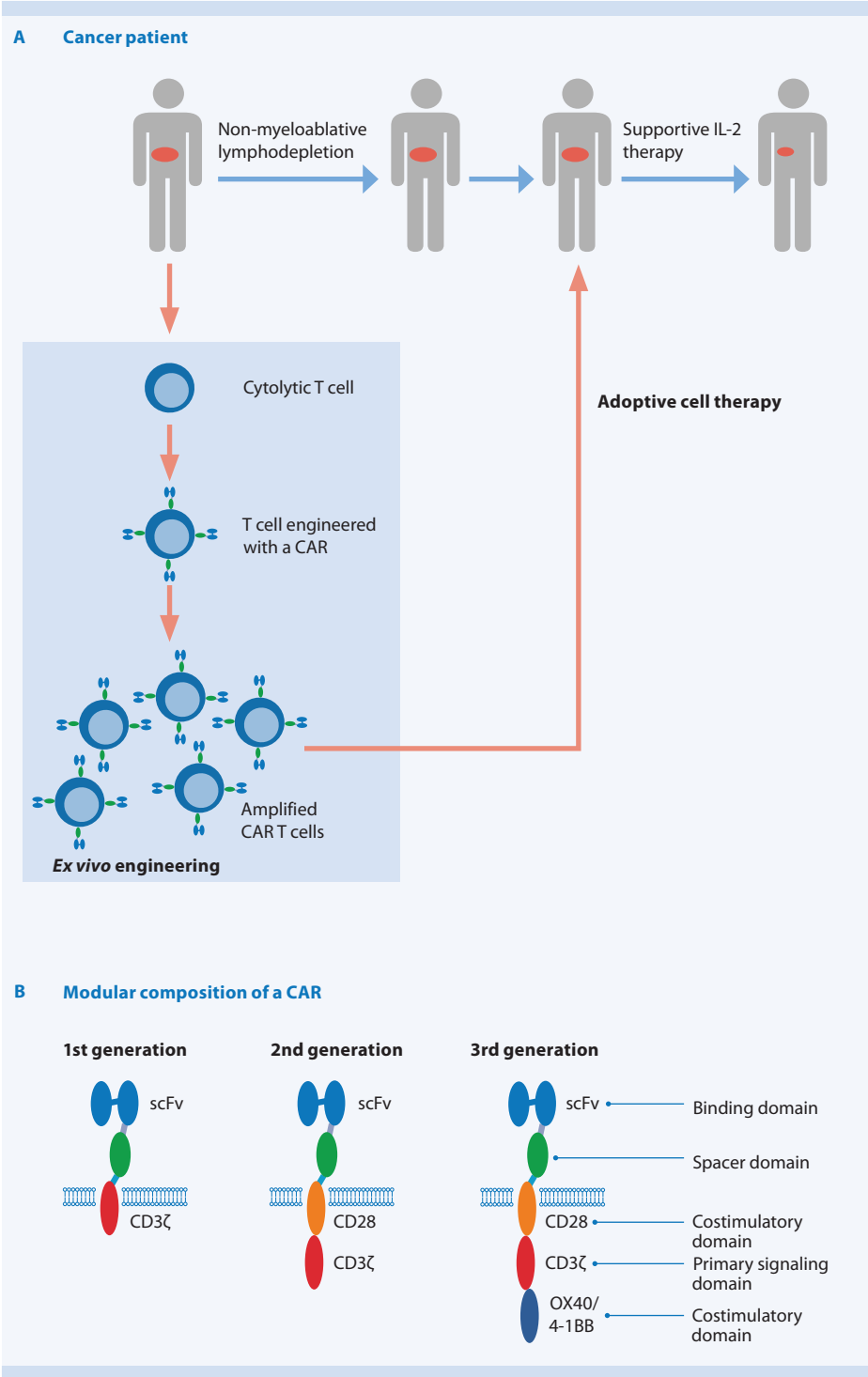


Figure 1 Adoptive cell therapy with chimeric antigen receptor (CAR)–modified T cells. (A) T cells taken from the cancer patient are *ex vivo* engineered with the chimeric antigen receptor (CAR), which recognizes a pre-defined target on the cancer cell. After adoptive transfer to the patient by transfusion, CAR T cells are expected to migrate to the tumor lesion, induce a pro-inflammatory reaction, and eliminate the cancer cells, resulting in a lasting tumor regression. (B) The CAR is composed of a single polypeptide chain. The extracellular single-chain fragment of variable region (scFv) antibody domain binds to the target antigen in an MHC-independent fashion. Upon CAR clustering, the intracellular CD3ζ chain, with or without costimulation through members of the CD28 family or the TNF-receptor family (4-1BB or OX40), initiates the downstream signaling for T cell activation.

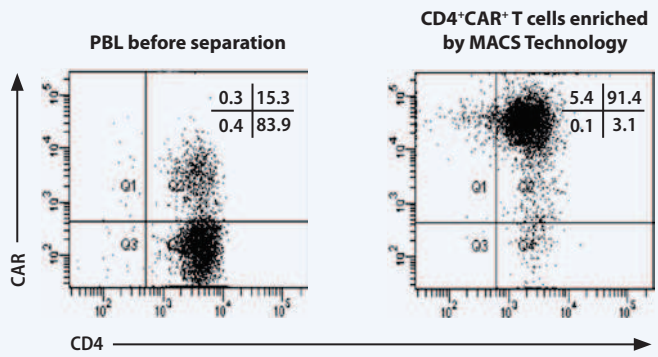


Figure 2 Engineered CAR T cells. CD4+ T cells were isolated from peripheral blood lymphocytes (PBL) using the CD4+ T Cell Isolation Kit (Miltenyi Biotec) and retrovirally transduced to express a CEA-specific CAR. Modified T cells were isolated by the autoMACS® Pro Separator after incubation with a PE-labeled anti-human IgG-Fc-antibody directed against the constant CAR spacer domain and PE-specific paramagnetic MicroBeads (Miltenyi Biotec). T cells were cultured for 6 days in the presence of 500 U/mL IL-2. CD4+CAR+ T cells were identified by anti-CD4-FITC and anti-human IgG-Fc-PE antibodies and analyzed by flow cytometry.

induced cell death, a second generation of CARs was designed, which in addition to CD3ζ harbor a costimulatory moiety mostly derived from CD28, OX40 (CD134), or 4-1BB (CD137), in the intracellular domain (fig. 1)[11]. Furthermore, CD28 was combined with other costimulatory domains, such as 4-1BB or OX40 in so-called third-generation CARs, which provide benefit for some T cell subsets[12–14].

CD28, the prototype of a family of costimulatory molecules, plays a physiological role on T cells by binding to the respective ligands on antigen-presenting cells (APCs) and augments the primary T cell activation signal provided by TCR CD3ζ. Since most cancer cells lack the physiological ligands for CD28, the CAR CD3ζ signaling domain was linked to the CD28 costimulatory domain within the same CAR[15–17]. Other costimulatory molecules of the TNF-receptor family, including 4-1BB (CD137) and OX40 (CD134), can also be used in the same way to improve CAR-mediated T cell activation. This type of CAR has the advantage that full T cell activation occurs in the absence of APCs. However, each costimulatory signal modulates the T cell effector function in a specific fashion[14].

CD28 sustains survival and prolongs polyclonal expansion of engineered T cells and induces IL-2 that is used in an autocrine fashion by redirected T cells to increase their amplification[18,18]. Thereby, the CD28-CD3ζ CAR counteracts transforming growth factor-β1 (TGF-β1)–mediated repression in T cell amplification[20]. Moreover, CD28 prevents activation-induced cell death, which, together with enhanced persistence and amplification, results in an improved anti-tumor attack.

Although there is some overlap, each costimulus modulates a distinct pattern of T cell activities. In-depth comparison of T cell effector functions revealed that CD28 and 4-1BB costimulation triggers T cell amplification more efficiently than OX40[14]. This has been observed for both CD4+ and CD8+ T cells. Noteworthy, IL-2 secretion is only induced upon CD28 costimulation, but not upon OX40 or 4-1BB, whereas CD28, OX40, and 4-1BB each increase IFN-γ secretion. The cytolytic activity, however, is not dramatically altered by OX40 or 4-1BB costimulation, but some increase is observed upon CD28 costimulation. In CD8+ T cells 4-1BB costimulation, and to a lesser extent CD28 and OX40 costimulation, prevents activation-induced cell death, while in CD4+ T cells cell death is diminished by either costimulation. ICOS, OX40, and 4-1BB, each combined with CD3ζ, improve clonal expansion and enhance effector functions to resting human T cells[21].

The matter of T cell subsets: different cells perform differently

Different T cell subsets are currently explored for use in CAR engineering to develop cell therapies. Detailed analyses *in vitro,* however, revealed that different subsets not only perform differently with respect to persistence, cytokine release, and anti-tumor activity, but also need different costimuli to become fully activated. By using the antibody-derived binding domain for target recognition, the MHC restriction is bypassed and both CD8+ and CD4+ T cell subsets can be specifically redirected[22–24]. Equipped with a CAR, CD4+ T cells showed a cytolytic potential similar to CD8+ T cells. Human CD8+ T cells predominantly use the perforin and granzyme pathway and to some extent Fas/Fas-ligand (Fas-L) or TNF/TNF-receptor (TNF-R) signaling to execute cytolysis. The mechanism of CAR-mediated lysis by redirected CD4+ T cells seems to be different in mouse and human. While murine CD4+ T cells mediate lysis by the death receptor system, human CD4+ T cells predominantly utilize granzyme/perforin. As a consequence, CAR-engineered CD4+ T cells derived from patient samples can not only efficiently provide help upon CAR-mediated activation, but also can directly eliminate tumor cells.

While engineered T cells adoptively transferred to patients are predominantly of young effector and central memory phenotype, repetitive antigen stimulation irreversibly triggers T cell maturation, giving rise to late memory cells with the KLRG-1+CD57+CD7−CCR7− phenotype in the long term. These cells preferentially accumulate in the periphery, are hypo-responsive upon CAR engagement and prone to activation-induced cell death. On the other hand, CCR7− CAR T cells persist in higher numbers in the tumor lesion although both the CCR7+ and CCR7− T cell subsets equally efficiently migrate to the tumor. A recent in-depth analysis revealed that the anti-tumor response of CCR7− CAR T cells is less efficient than that of CCR7+ T cells when redirected by a CD28-CD3ζ CAR. However, the anti-tumor response is restored by simultaneous CD28 and OX40 stimulation[25]. This is due to the fact that CCR7− T cells are prone to spontaneous and activation-induced

cell death, which is insufficiently prevented by CD28 but requires additional OX40 signaling.

Cytokine-induced killer (CIK) cells also raised interest for the use in such cell therapy as they have an extraordinary capability to recognize and destroy cancer cells in an HLA-independent fashion. The anti-tumor attack is further improved through redirecting CIK cells by a CAR[26,27]. CIK cells, however, require different activation signals than "young" T cells. CD28-CD3ζ CAR CIK cells displayed a superior anti-tumor capacity compared to "super-costimulation" by the CD28-CD3ζ-OX40 CAR, which rather accelerated terminal maturation and activation-induced cell death. CD28-CD3ζ CAR–mediated CIK activation also increased antigen-independent NKG2D-mediated lysis making such modified CIK cells potent tumor killers which deserve clinical exploration.

Despite these recent advances, experience with CAR-engineered primary NK cells and their clinical application in adoptive cell therapy is still limited. CD3ζ, CD28-CD3ζ, and 4-1BB-CD3ζ chain CARs were used to redirect NK cells towards defined targets, including CD19, CD20, HER2/neu (ErbB2), EpCAM, and GD-2[28–33]. In contrast to T cells, however, NK cells cannot provide IL-2, which is needed in an autocrine fashion for sustained amplification; co-expression of IL-15 bypassed this limitation[34]. Clinically applicable established NK cell lines, such as NK-92, circumvent the need to modify patient's autologous NK cells *ex vivo* and allow the generation of CAR-modified cells from a bulk of unmodified cells[35]. Such modified cell lines, which were irradiated prior to application to prevent permanent engraftment, were proven safe, and clinical responses were achieved in some patients[36].

CAR T cell therapy resulted in tumor regression in recent early phase trials

Current clinical protocols in adoptive cell therapy stipulate that patient's T cells are modified *ex vivo* by retro- or lentiviral gene transfer to express the respective CAR, amplified to therapeutically relevant numbers, and transfused back to the patient who is pre-treated according to a non-myeloablative lymphodepleting regimen, i.e., patients receive a cyclophosphamide pre-treatment for lymphodepletion followed by infusion of engineered T cells[37]. The immunomodulatory effect of cyclophosphamide in this context is probably multifactorial, and several, mutually non-exclusive mechanisms may play a role, including i) enhanced homeostatic expansion of modified T cells with the help of a niche created by the pre-treatment, ii) induction of T cell growth factors, including type I interferons, iii) stimulation of innate immune cells, particularly dendritic cells, and iv) elimination of Treg cells.

An increasing number of clinical trials are currently evaluating second- and third-generation CARs in the treatment of malignant diseases, mostly addressing leukemia and lymphoma[38–41]. CD19-specific CAR T cells induced complete and lasting remission of refractory $CD19^+$ B cell chronic lymphocytic leukemia (CLL) in all of the first three reported patients. CAR T cells substantially expanded compared to the initial blood level, persisted for at least 6 months, and were effective in an anti-tumor response even at low dosage levels of about 1.5×10^5 cells per kg[40]. The persistence of CAR-modified T cells in these trials is likely due to the costimulation sustaining T cell survival in the long term and the repetitive re-stimulation by $CD19^+$ healthy B cells, which are also targets for the anti-CD19 CAR T cells. The T cell anti-tumor response was accompanied by an increase in the pro-inflammatory cytokines IFN-γ and IL-6, which paralleled the clinical symptoms of a "cytokine storm" and was reduced by application of an IL-6–neutralizing antibody[41]. The same CAR is currently being evaluated in the treatment of pediatric $CD19^+$ acute leukemia, in some cases with spectacular success[42].

The specificity of redirected T cells is defined by the CAR targeting antibody. Therefore, healthy cells expressing the target antigen are likewise eliminated, as seen during CAR T cell therapy of $CD19^+$ lymphoma, which is accompanied by sustained B cell depletion[38,40]. Such "on-target off-organ" activation of CAR T cells also occurred when HER2/neu (ErbB2) was targeted, which is ubiquitously expressed on healthy tissues[43]. In addition, CARs of the current design can cause anaphylaxis in humans, most likely through IgE antibodies specific to the CAR, which harbors a murine antigen binding domain[44]. Despite these and other observed severe adverse events, MHC-independent targeting of cancer cells by CAR-modified T cells showed promise in controlling at least leukemia in the long term. Trials in the near future will address whether solid cancer lesions can also be successfully treated using CAR T cells.

Perspectives for the future of therapies based on CAR-redirected T cells

While first clinical success is sustaining hope that cell therapy with CAR-redirected T cells may cure leukemia in the long term, a number of issues need to be addressed with regard to technical aspects and targets when treating other malignant diseases.

It is still a challenge to obtain relevant numbers of CAR-engineered T cells with a favorable phenotype for clinical application. T cells optimally grow in rather low cell densities, i.e., 10^6 cells per mL in static cultures; higher cell numbers for clinical application require advanced non-static systems[45]. In order to translate the current experimental application into clinical routine procedures, it would be beneficial to manufacture the cells in a closed system from the very beginning until the final cell product. Such a process, performed in a closed device and in a fully automated fashion, would allow for full standardization of the procedure according to current guidelines. Moreover, such a device would enable the simultaneous production of multiple batches within the same cleanroom. The development of closed systems is in its final stage, and the systems are expected to enter clinical application in the near future.

Engineered T cells need to be active in solid tumor tissues over a prolonged period of time in order to fully execute their anti-tumor activity; solid tumor lesions, however, often build up an immunosuppressive micro-environment, which needs to be overcome. Several strategies to avoid T cell repression are currently under investigation. Upon repetitive restimulation, T cells convert to effector memory cells and lose responsiveness to standard CD28-CD3ζ CAR signaling. Combined CD28-OX40 costimulation is required to rescue those

cells from activation-induced cell death[25]. Additional costimulation by 4-1BB may also provide benefit[46].

A prerequisite for extensive T cell expansion is thought to be non-myeloablative lymphodepleting pre-conditioning followed by IL-2 administration to sustain T cell expansion; other cytokines, such as IL-7 and IL-15 are currently also explored. Alternatively, CAR T cells are locally applied into the tumor lesion by puncture or endoscopy with only limited diffusion within the following days. The strategy is currently evaluated in the treatment of head-and-neck cancer (EudraCT 2012-001654-25, NCT01722149) and will be shortly applied to the treatment of cutaneous lymphoma (EudraCT 2011-003125-10).

An elegant solution of providing T cell responses in the long term is the use of virus-specific T cells, which obtain required costimulatory signals when engaging virus-infected cells by their TCR. Current trials use EBV- or CMV-specific, autologous T cells engineered with a first- or second-generation CAR, for instance directed against HER2/neu (ErbB2) (NCT01109095), CD30 (NCT01192464), CD19 (NCT00709033; NCT01475058; NCT01430390; NCT00840853; NCT01195480), or GD-2 (NCT00085930). Moreover, the strategy takes advantage of the virus-specific T cell's capacity to amplify and pose only a low risk of inducing graft-versus-host disease.

References

1. Morgan, R.A. *et al.* (2006) Science 314: 126–129.
2. Gross, G. *et al.* (1989) Proc. Natl. Acad. Sci. USA 86: 10024–10028.
3. Eshhar, Z. (2008) Handb. Exp. Pharmacol. 181: 329–342.
4. Hombach, A. *et al.* (1997) Gastroenterology 113: 1163–1170.
5. Mezzanzanica, D. *et al.* (1998) Cancer Gene Ther. 5: 401–407.
6. Westwood, J.A. *et al.* (2005) Proc. Natl. Acad. Sci. USA 102: 19051–19056.
7. Stewart-Jones, G. *et al.* (2009) Proc. Natl. Acad. Sci. USA 106: 5784–5788.
8. Till, B.G. *et al.* (2008) Blood 112: 2261–2271.
9. Kershaw, M.H. *et al.* (2006) Clin. Cancer Res. 12: 6106–6115.
10. Lamers, C.H. *et al.* (2006) J. Clin. Oncol. 24: e20-e22.
11. Hombach, A.A. *et al.* (2013) Curr. Mol. Med. 13: 1079–1088.
12. Zhao, Y. *et al.* (2009) J. Immunol. 183: 5563-5574.
13. Carpenito, C. *et al.* (2009) Proc. Natl. Acad. Sci. USA 106: 3360–3365.
14. Hombach, A.A. and Abken, H. (2011) Int. J. Cancer 129: 2935–2944.
15. Hombach, A. *et al.* (2001) J. Immunol. 167: 6123–6131.
16. Thislethwaite, F. *et al.* (2005) Curr. Opin. Mol. Ther. 7: 48–55.
17. Finney, H.M. *et al.* (1998) J. Immunol. 161: 2791–2797.
18. Beecham, E.J. *et al.* (2000) J. Immunother. 23: 631–642.
19. Hombach, A. *et al.* (2001) Cancer Res. 61: 1976–1982.
20. Koehler, H. *et al.* (2007) Cancer Res. 67: 2265–2273.
21. Finney, H.M. *et al.* (2004) J. Immunol. 172: 104–113.
22. Hombach, A. *et al.* (2006) J. Immunol. 177: 5668–5675.
23. Hombach, A. *et al.* (2001) J. Immunol. 167: 1090–1096.
24. Moeller, M. *et al.* (2007) Cancer Res. 67: 11428–11437.
25. Hombach, A.A. *et al.* (2013) Hum. Gene Ther. 24: 259–269.
26. Tettamanti, S. *et al.* (2013) Br. J. Haematol. 161: 389–401.
27. Hombach, A.A. *et al.* (2013) Mol. Ther. 21: 2268–2277.
28. Boissel, L. *et al.* (2009) Leuk. Res. 33: 1255-1259.
29. Müller, T. *et al.* (2008) Cancer Immunol. Immunother. 57: 411–423.
30. Kruschinski, A. *et al.* (2008) Proc. Natl. Acad. Sci. USA 105: 17481–17486.
31. Esser, R. *et al.* (2012) J. Cell. Mol. Med. 16: 569–581.
32. Imai, C. *et al.* (2005) Blood 106: 376–383.
33. Altvater, B. *et al.* (2009) Clin. Cancer Res. 15: 4857–4866.
34. Sahm, C. *et al.* (2012) Cancer Immunol. Immunother. 61: 1451–1461.
35. Klingemann, H.G. (2013) Cytotherapy 15: 1185–1194.
36. Tonn, T. *et al.* (2013) Cytotherapy 15: 1563–1570.
37. Dudley, M.E. *et al.* (2005) J. Clin. Oncol. 23: 2346–2357.
38. Brentjens, R. *et al.* (2010) Mol. Ther. 18: 666-668.
39. Kalos, M. *et al.* (2011) Sci. Transl. Med. 3: 95ra73.
40. Porter, D.L. *et al.* (2011) N. Engl. J. Med. 365: 725–733.
41. Maus, M.V. *et al.* (2014) Blood 123: 2625–2635.
42. Grupp, S.A. *et al.* (2013) N. Engl. J. Med. 368: 1509-1518.
43. Morgan, R.A. *et al.* (2010) Mol. Ther. 18: 843–851.
44. Maus, M.V. *et al.* (2013) Cancer Immunol. Res. 1: 26–31.
45. Vera, J.F. *et al.* (2010) J. Immunother. 33 : 305–315.
46. Song, D.G. *et al.* (2011) Cancer Res. 71: 4617–4627.

Conflict of interest statement

The authors declare that the research in the author's laboratory was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

Work in the author's laboratory is supported by the Deutsche Forschungsgemeinschaft, Deutsche Krebshilfe, the Wilhelm Sander-Stiftung, the European Union (European Regional Development Fund – Investing in your future) and the German federal state North Rhine-Westphalia (NRW), and the Fortune program of the Medical Faculty of the University of Cologne.

Miltenyi Biotec

The MACSQuant® Tyto™

The revolution in cell sorting has begun

The MACSQuant® Tyto™ is revolutionizing cell sorting. Our patented microchip-based technology opens new possibilities in basic research and clinical settings with high-speed multiparameter flow sorting in the safety of a fully enclosed cartridge.

Innovation
Sort cells with the world's fastest mechanical sort valve and 11-parameter fluorescence-based sorting.

Safety
Samples and operator are kept contamination-free and safe with disposable, fully enclosed cartridges.

Viability
Cells are gently driven through the microchip with low positive pressure. Less stress means higher yield of viable, functional cells.

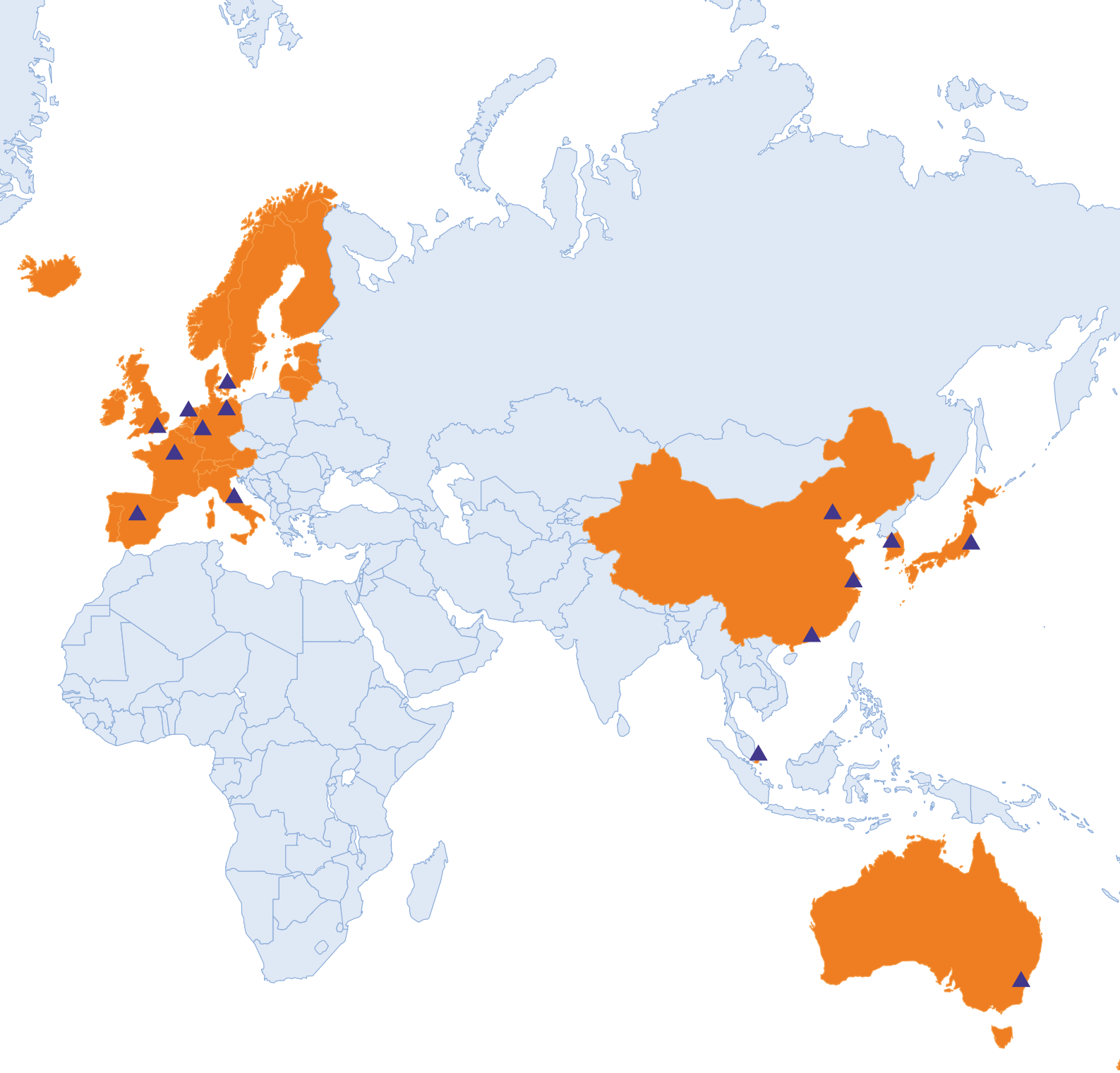
Ease of use
No droplet delay or laser alignment needed. Simply insert the cartridge, gate on cells, and sort.

▶ **miltenyibiotec.com/tyto**

The MACSQuant Tyto is for research use only.

25 Years of Miltenyi Biotec

For a quarter century, Miltenyi Biotec has helped brilliant minds advance scientific discovery and cellular therapies. On our 25th anniversary, we look back at the milestones that got us where we are today, and the breakthroughs that inspire us for tomorrow.



1989

Miltenyi Biotec is founded

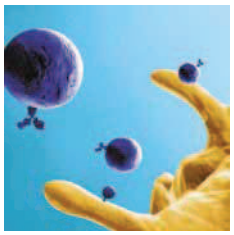
The magnetic cell separation technology developed by Stefan Miltenyi provides the firm basis for Miltenyi Biotec and would become the industry standard.

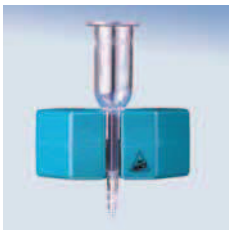
Germany – Bergisch Gladbach
Historical view

1990

MACS® Technology

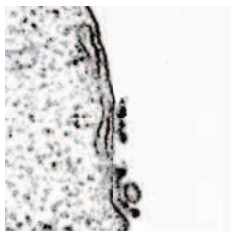
The groundbreaking method of separating cells using nano-sized MicroBeads, columns, and magnetic separators is patented.





Since 1989, Miltenyi Biotec has continued to grow. At its headquarters in Germany, new buildings and facilities reflect this exciting expansion.

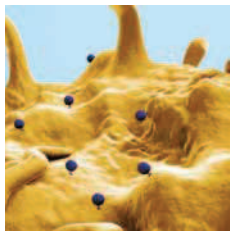
Germany – Bergisch Gladbach
Headquarters



1992

Expansion to the U.S.

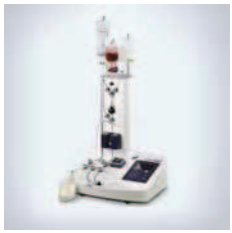
Miltenyi Biotec expands to North America with its U.S. headquarters in Northern California, and would later go on to open offices in other biotechnology hubs, Boston and San Diego.



1992

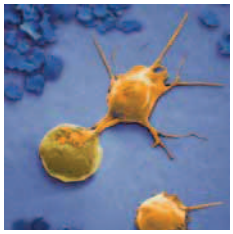
CliniMACS® System

A joint venture with Amgen is formed to develop the technology that would become the basis for the industry-changing CliniMACS System.



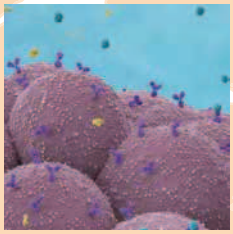
1995

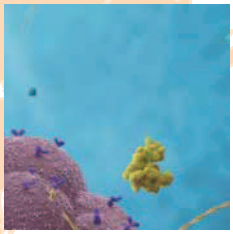
Miltenyi Biotec opens the UK subsidiary in Bisley, Surrey, just outside of London.



1ST SALES REP IN GERMANY

Expansion to the UK

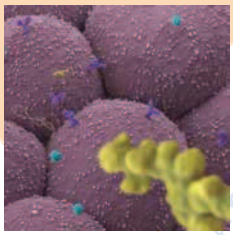




2002

GMP fa opens

>300 G WEIGHT OF OUR PRINTED PRODUCT CATALOG



Miltenyi Bio purchases a in Germany devices, co

1997

CE marking in Europe

>500 PRODUCTS AVAILABLE

The CliniMACS System receives its CE marking as an approved medical device in Europe.

1998

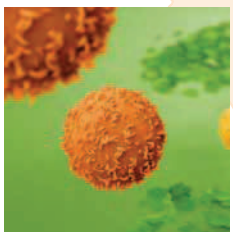
Expansion to Southern Europe

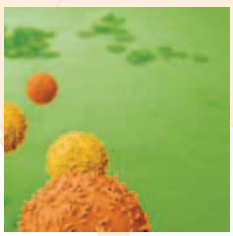
2001

Expansion to Asia-Pacific

>80 WEI OF OUR PROD CATA

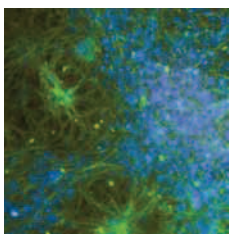
New subsidiaries in France, Italy, and Spain enlarge the Miltenyi Biotec family.

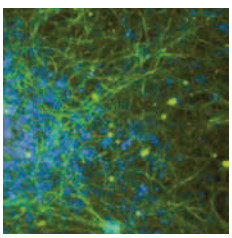




Miltenyi Biotec opens its first Asia subsidiary in China, and would later go on to open subsidaries in Japan, Singapore, Australia, and South Korea.

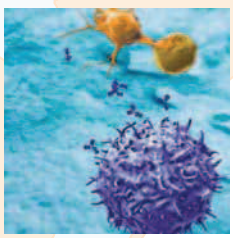
20

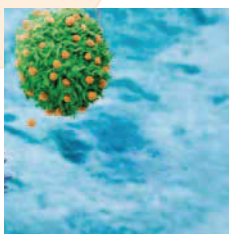




1999

autoMACS® Separator

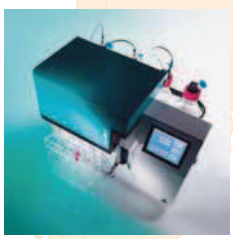


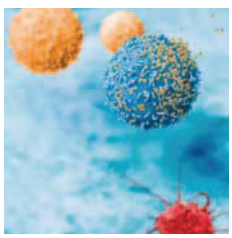


Miltenyi Bio moves to B the Netherl and Luxem foster futur especially in of cellular th

200 EMPLOYEES REACHED

Miltenyi Biotec launches the autoMACS Separator – at that time the only automated magnetic cell separator.





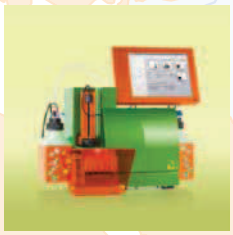
cility

otec aquires PlasmaSelect and
a state-of-the-art manufacturing facility
y for the production of GMP-compliant
nsumables, and reagents.

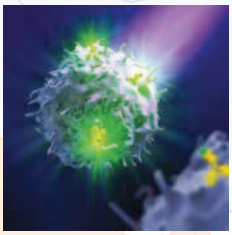
2008

Miltenyi Biotec introduces a new design approach to flow cytometers with easy-to-use instruments for researchers at all levels.

MACSQuant® Flow Cytometers



10,000 PATIENTS TREATED USING OUR CLINIMACS SYSTEM



2012

The Nordic subsidiary is opened in Sweden, covering Scandinavia, the Baltic countries, and Iceland.

800 EMPLOYEES REACHED

2006

Miltenyi Biotec receives the Frost & Sullivan award in London for the PrepProtect™ Stabilization Buffer.

Product Innovation of the Year

MORE THAN 28,500 CE-MARKED CLINIMACS REAGENT VIALS PRODUCED

2009

gentleMACS™ Dissociator

1,000 EMPLOYEES REACHED

1,359,617 REAGENT KITS PRODUCED SINCE 1995

00 G GHT PRINTED DUCT ALOG

05

Expansion Benelux

tec makes
elgium,
ands
bourg to
e growth
n the field
herapy.

2006

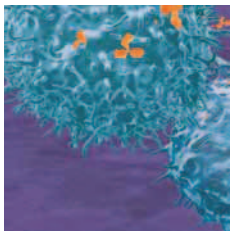
Launch of the autoMACS Pro Separator, the most advanced automated cell separation instrument on the market.

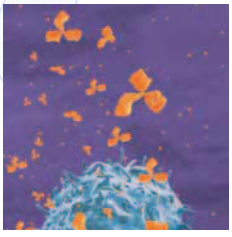
autoMACS® Pro Separator

> 1,750 G WEIGHT OF OUR PRINTED PRODUCT CATALOG

The acquisition of the Swiss company Medic Tools leads to the development of the successful gentleMACS Dissociator instruments.

100 CLINIMACS INSTRUMENTS SOLD IN THE US





2012

This GMP-compliant instrument offers advanced solutions for automating cell-processing workflows.

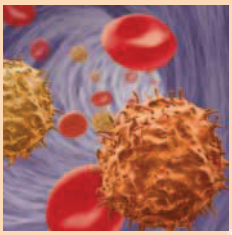
1,000
AUTOMACS PRO SEPARATORS SOLD

200
GMP CERTIFICATES

25
YEARS

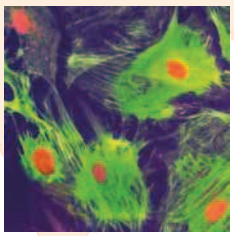
Expansion to Northern Europe

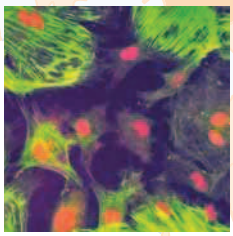
1,400
EMPLOYEES REACHED



35,000
CLINICAL CELL SEPARATIONS PERFORMED USING OUR DEVICES

MACS
Miltenyi Biotec





3,000
RESEARCH TOOLS

2014

FDA approval

1 FDA APPROVAL FOR THE CLINIMACS CD34 REAGENT SYSTEM

2013

MACSQuant® Tyto™

Miltenyi Biotec receives its first FDA approval, for the CliniMACS CD34 Reagent System.

CliniMACS Prodigy®

Miltenyi Biotec acquires Owl biomedical and develops the revolutionary microchip-based flow sorting instrument, MACSQuant Tyto.

OFFICES IN 25 COUNTRIES


Germany – Bergisch Gladbach Headquarters

7,000
PRODUCTS AVAILABLE

2014

Lentigen Technology

CITED IN MORE THAN 20,000 PUBLICATIONS

CITED IN MORE THAN
300
CLINICAL PUBLICATIONS

2,000
PUBLICATIONS CITING AUTOMACS CELL SEPARATION

Miltenyi Biotec acquires Lentigen Technology Inc., a global leader in lentiviral technology for cell and gene therapy applications.

Lentigen Technology Inc.
a Miltenyi Biotec Company

MACS&more (ISSN 1610-4994) is published by Miltenyi Biotec GmbH.

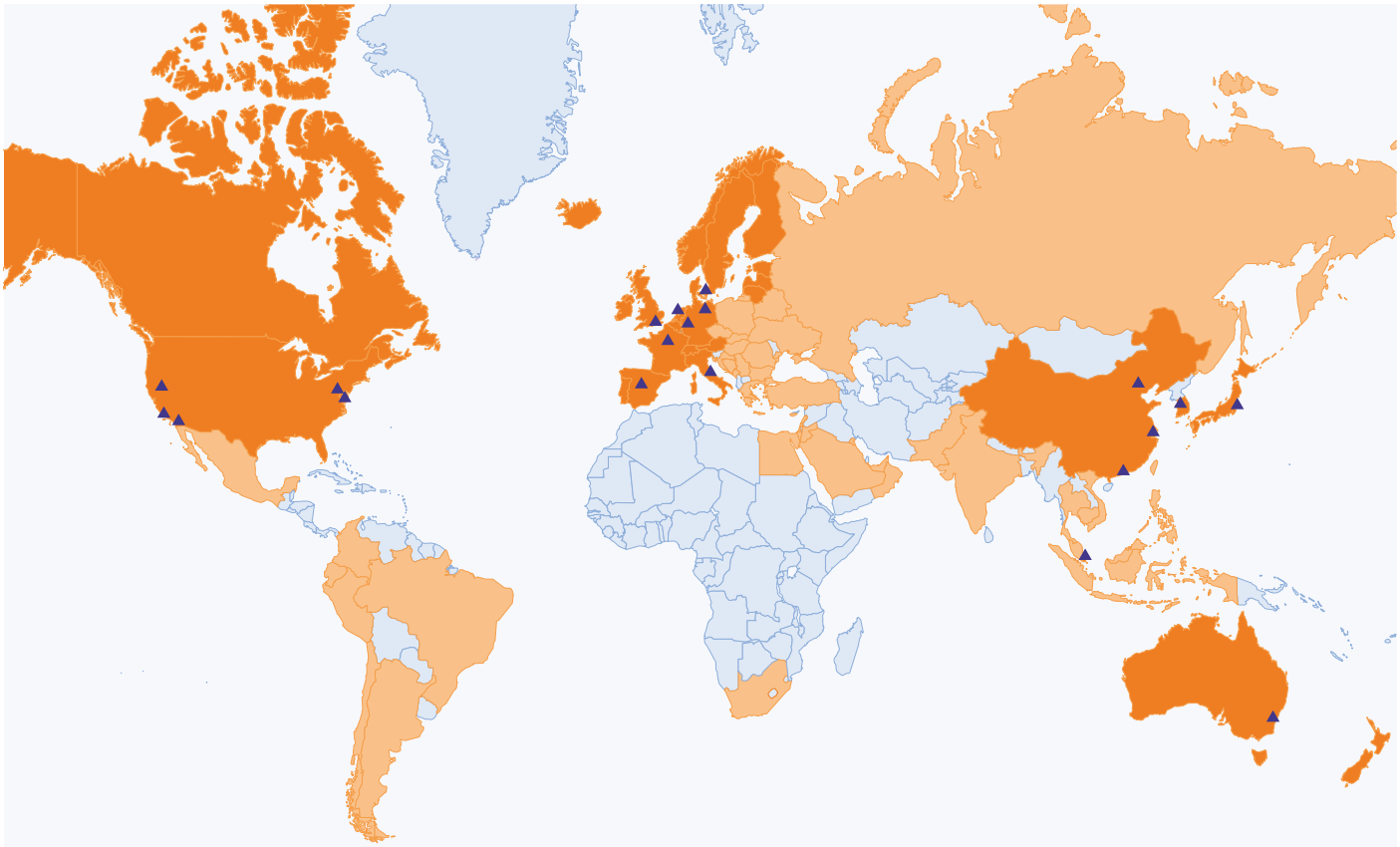
Editorial board: Volker Arps, Mario Assenmacher, Dirk Balshüsemann, Andreas Bosio, Vincent Chu, Steven Dublin, Olaf Hardt, Guy Hewlett, Christoph Hintzen, Sebastian Knöbel, Bianca Lippert, Alexander Scheffold, Jürgen Schmitz, Jacqueline Sutter
Editors: Stefan Miltenyi, Ralph Schaloske
Graphics & Layout: Miltenyi Biotec GmbH, Graphics team

Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68,
51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0
macs@miltenyibiotec.de

The CliniMACS® System components, including Reagents, Tubing Sets, Instruments, and PBS/EDTA Buffer, are manufactured and controlled under an ISO 13485–certified quality system. In the EU, the CliniMACS System components are available as CE-marked medical devices. In the US, the CliniMACS CD34 Reagent System, including the CliniMACS Plus Instrument, CliniMACS CD34 Reagent, CliniMACS Tubing Sets TS and LS, and the CliniMACS PBS/EDTA Buffer, is FDA approved; all other products of the CliniMACS Product Line are available for use only under an approved Investigational New Drug (IND) application or Investigational Device Exemption (IDE). In the US, the components of the CliniMACS Prodigy® System are for research use only and not for human therapeutic or diagnostic use. CliniMACS MicroBeads are for research use only and not for human therapeutic or diagnostic use.

MACS GMP Products are for research use and *ex vivo* cell culture processing only, and are not intended for human *in vivo* applications. For regulatory status in the USA, please contact your local representative. MACS GMP Products are manufactured and tested under a certified ISO 9001 quality system and in compliance with relevant GMP guidelines. They are designed following the recommendations of USP <1043> on ancillary materials. No animal- or human-derived materials were used for manufacture of these products.

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use.



Germany/Austria/ Switzerland
Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68
51429 Bergisch Gladbach
Germany
Phone +49 2204 8306-0
Fax +49 2204 85197
macs@miltenyibiotec.de

USA/Canada
Miltenyi Biotec Inc.
2303 Lindbergh Street
Auburn, CA 95602, USA
Phone 800 FOR MACS
Phone +1 530 888 8871
Fax +1 530 888 8925
macs@miltenyibiotec.com

Australia
Miltenyi Biotec
Australia Pty. Ltd.
Unit 16A, 2 Eden Park Drive
Macquarie Park NSW 2113
Australia
Phone +61 2 8877 7400
Fax +61 2 9889 5044
macs@miltenyibiotec.com.au

Benelux
Miltenyi Biotec B.V.
Schipholweg 68 H
2316 XE Leiden
The Netherlands
macs@miltenyibiotec.nl
Customer service The Netherlands
Phone 0800 4020120
Fax 0800 4020100
Customer service Belgium
Phone 0800 94016
Fax 0800 99626
Customer service Luxembourg
Phone 800 24971
Fax 800 24984

China
Miltenyi Biotec Technology & Trading (Shanghai) Co., Ltd.
Room 2309
No. 319, Xianxia Road
Changning District
200051 Shanghai, P.R. China
Phone +86 21 62351005
Fax +86 21 62350953
macs@miltenyibiotec.com.cn

France
Miltenyi Biotec SAS
10 rue Mercoeur
75011 Paris, France
Phone +33 1 56 98 16 16
Fax +33 1 56 98 16 17
macs@miltenyibiotec.fr

Italy
Miltenyi Biotec S.r.l.
Via Persicetana, 2/D
40012 Calderara di Reno (BO)
Italy
Phone +39 051 6 460 411
Fax +39 051 6 460 499
macs@miltenyibiotec.it

Japan
Miltenyi Biotec K.K.
Nittsu-Eitai Building 5F
16-10 Fuyuki, Koto-ku,
Tokyo 135-0041, Japan
Phone +81 3 5646 8910
Fax +81 3 5646 8911
macs@miltenyibiotec.jp

Nordics and Baltics
Miltenyi Biotec Norden AB
Scheelevägen 17
223 70 Lund
Sweden
macs@miltenyibiotec.se
Customer service Sweden
Phone 0200-111 800
Fax 046-280 72 99
Customer service Denmark
Phone 80 20 30 10
Fax +46 46 280 72 99
Customer service Norway, Finland, Iceland, and Baltic countries
Phone +46 46 280 72 80
Fax +46 46 280 72 99

Singapore
Miltenyi Biotec Asia Pacific Pte Ltd.
100 Beach Road
#28-06 to 28-08 Shaw Tower
Singapore 189702
Phone +65 6238 8183
Fax +65 6238 0302
macs@miltenyibiotec.com.sg

South Korea
Miltenyi Biotec Korea
Petra building
659 Yeoksam-dong
Gangnam-gu
Seoul, South Korea
Phone +82 2 555 1988
Fax +82 2 555 8890
macs@miltenyibiotec.co.kr

Spain
Miltenyi Biotec S.L.
C/Luis Buñuel 2
Ciudad de la Imagen
28223 Pozuelo de Alarcón (Madrid)
Spain
Phone +34 91 512 12 90
Fax +34 91 512 12 91
macs@miltenyibiotec.es

United Kingdom
Miltenyi Biotec Ltd.
Almac House, Church Lane
Bisley, Surrey GU24 9DR, UK
Phone +44 1483 799 800
Fax +44 1483 799 811
macs@miltenyibiotec.co.uk

www.miltenyibiotec.com

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

Cover image: The illustration shows T cells attached to a tumor cell. Certain $CD8^+$ T cells can specifically recognize tumor antigens on the cell surface and have the capacity to kill tumor cells directly.

130-106-199