Project Final Report

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Colour legend:

Italics: explanation about text / charts

Yellow: to be provided by GABO:mi

Green: to be provided by the coordinator

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Patients with high-risk hematological tumors can be cured by allogeneic hematopoietic stem cell transplantation (HSCT). However, the main causes of failure of HSCT are infections, tumor relapse and over-shooting immune responses of the donor T cells to healthy cells and tissues of the patient or graft-versus host disease (GvHD). Therefore, this treatment is still associated with a high morbidity and mortality, as well as a high economic burden.

The main objectives of T-Control were to develop **two Streptamer-based GMP** (good manufacturing practice) **compliant cell selection processes and their corresponding cell products** within a three-year project duration. The products are a multi virus- and a tumor-specific CD8+ T cell product ("multi specific T-cell product"), to boost immune function post-transplant for the treatment of infections and residual/recurrent tumor cells after HSCT, and a pT-reg product to suppress the immune function post-transplant for the treatment of GvHD after HSCT. The rationale for developing this product was the urgent medical need to provide treatment options for the two main causes of allogeneic HSCT failure (insufficient immune response to viruses and tumors). This product was successfully developed also in the context of GvHD. Both cell products were generated using the same cell selection technology (Streptamer technology), but with different aim, namely to meet these two different, major clinical needs. One product was shown to boost insufficient immune responses to infections and residual tumor and the other was developed to suppress overshooting immune responses causing GvHD to improve the outcome of allogeneic HSCT in general.

T-Control consortium was able to produce the multi-antigen-specific T cell product of high purity and with a sufficient number of multi-antigen specific T cells for the vast majority of patients. 35 patients (planned 34 patients) were included in the trial but not all the patients received the planned T cell product. Thus we will continue recruitment. The T cell depletion – either by Campath in the bag or by in vitro T cell depletion using CD34 selection significantly reduced the incidence and severity of acute GvHD in spite of the delayed infusion of mutli-antigen-specific T cell products obtained from the stem cell donor. Further follow up will have to show whether also cGvHD could be reduced. The analysis of the clinical data we obtained until now indicated that there is no increase in viral infections inspite of the fact that the patients included in the trial received a T cell depleted allograft. Further data on virus-specific t cell reconstitution will be generated to correlate the pathogen-specific T cell reconstitution with the recurrence rate of viral infections post-transplant (following this novel transplantation procedure). Longer follow up and additional patient inclusion will be required to analyze the impact of T cell depletion of the allograft and the add back of TAA- and MiHAg-specific T cells after alloSCT on relapse rate and reconstitution of tumor-reactive T cells.

Due to the hurdles occurred during the generation of cord blood derived pT-regs by using the Streptamer technology, and also from adult peripheral blood as way-out strategy, the new objective of T-Control was to further develop the pT-reg product but not to initiate a clinical trial. This goal could be achieved and we have a clinical product (streptamer-selected pT-regs with ex vivo expansion) available and have already designed a clinical trial to use this product (for patients with steroid-refractory GvHD will receive the in vitro expanded and activated pT-regs from cord blood units and receive low dose IL-2 for further in vivo expansion and activation).

T-Control

Logo: T-CONTROL

Project title: Donor T cells for Immune Control

Website: www.t-control.info

Contractors involved (T-Control consortium):

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1.1 Executive summary

Patients with high-risk hematological tumors can be cured by allogeneic hematopoietic stem cell transplantation (HSCT). However, the main causes of failure of HSCT are infections, tumor relapse and over-shooting immune responses of the donor T-cells to healthy cells and tissues of the patient or graft-versus host disease (GvHD). Therefore, this treatment is still associated with a high morbidity and mortality, as well as a high economic burden.
The main objectives of T-Control were to develop two Streptamer-based GMP (good manufacturing practice) compliant T-cell selection processes and their corresponding cell products within a three-year project duration. The products are a multi virus- and a tumor-specific CD8+ T-cell product ("multi specific T-cell product"), to boost immune function post-transplant for the treatment of infections and residual/recurrent tumor cells after HSCT, and a pT-reg product to suppress the immune function post-transplant for the treatment of GvHD after HSCT. The rationale for developing this product was the urgent medical need to provide treatment options for the two main causes of allogeneic HSCT failure (insufficient immune response to viruses and tumors and severe acute and/or chronic graft versus host disease). This product was successfully developed also in the context of GvHD. Both cell products were planned to be generated using the same cell selection technology (Streptamer technology), but with different aim, namely to meet these two different, major clinical needs. One product should be used to boost insufficient immune responses to infections and residual tumor and the other should suppress overshooting immune responses causing GvHD to improve the outcome of allogeneic HSCT in general.
Based on the results generated so far the T-Control consortium will achieved the first objective, i.e. to produce a multi antigen specific T-cell product under GMP conditions at 2 different sites and finalisation of the respective clinical trial. Due to the hurdles occurred during the generation of cord blood derived pT-regs by using the Streptamer technology, and also from adult peripheral blood as way-out strategy, the new objective of T-Control to develop the pT-reg product but not to initiate a clinical trial would be also achieved.

1.2 Summary description of project context and objectives:

Background and Aims

Patients with high-risk hematological tumors can be cured by allogeneic hematopoietic stem cell transplantation (HSCT). However, the main causes of failure of HSCT are infections, tumor relapse and over-shooting immune responses of the donor T cells to healthy cells and tissues of the patient or graft-versus host disease (GvHD). Therefore, this treatment is still associated with a high morbidity and mortality, as well as a high economic burden.

The previous EU FP6 project, ALLOSTEM, resulted in a number of achievements, including a 'first in man' clinical trial that used a highly innovative novel cell selection strategy (Streptamer technology) for the generation of non-ATMP (advanced therapy medicinal product) monovirus specific T cell products. Continuing from ALLOSTEM, the main objectives of T-Control were to develop **two *Streptamer-based GMP*** *(good manufacturing practice)* ***compliant cell selection processes and their corresponding*** **cell products** within a three-year project duration. The products are a multivirus- and a tumor-specific CD8+ T cell product ("multispecific T-cell product"), to boost immune function post-transplant for the treatment of infections and residual/recurrent tumor cells after HSCT, and a pT-reg product to suppress the immune function post-transplant for the treatment of GVHD after HSCT. The rationale for developing these two products was the urgent medical need to provide treatment options for the two main causes of allogeneic HSCT failure (insufficient immune response to viruses and tumors and overshooting immune responses in the context of GVHD). Both cell products were planned to be generated using the same cell selection technology (Streptamer technology), but with different aim, namely to meet these two different, major clinical needs. One product should be used to boost insufficient immune responses to infections and residual tumor and the other should suppress overshooting immune responses causing GVHD to improve the outcome of allogeneic HSCT in general.

Work strategy and general description

The basic material- and knowledge-flow comprises JUNO´s production of Streptamer reagents for the multispecific T cell product and pT-reg cell product, and their delivery to LUMC respectively. LUMC developed a selection process and quality control procedures for the respective cell product. This know- how has been transferred to JUNO to

enable JUNO to establish clinical grade selection sets for the developed processes. In addition this know-how has been ransferred to UKW for generating the documentation for the clinical trials. JUNO validated the selection kits for GMP compliant selection procedures and utilize these selection methods for the production of the therapeutic cell products at the GMP unit of the Institute for Transfusion Medicine and Immunohaematology Frankfurt/Main, German Red Cross Blood Donor Service (DRK-BSD) in Frankfurt and in the LUMC facilities.

The first task performed by JUNO (MHC-Streptamer production, WP5) in cooperation with LUMC (development of the multispecific T cell product, WP1) and with AN (development of the pT-reg product, WP2). For the multispecific T cell product, JUNO produced between 8-12 MHC-Streptamers per HLA-allele (WP5). LUMC tested different compositions of these Streptamers for their ability to optimally select the corresponding T cell mixtures from PBMs. Subsequently, the efficacy of the cell products to control infection and relapse have been analyzed in appropriate test systems (WP1).

After completion of the relevant Streptamer development, JUNO established a Streptamer reagent production (WP5) for continuous supply for the clinical trial, which is led by UKW (WP3). The non- randomized phase I/II trial of the Streptamer-selected multispecific cell product included 13 patients to demonstrate statistically relevant effects. Primary endpoints addressed the safety profile of the cell therapeutics whereas secondary endpoints addressed efficacy of the cell products (WP3).

Management structure and procedures

The Project Coordinator ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. He submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of ART he was responsible for the proper use of funds and their transfers to participants. The T-Control office was established by and based at the coordinator in Würzburg and at ART in Munich. The Project Office at the Coordinator was concerned with the scientific management and the co-ordination of all research activities. The Project Office at ART was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The General Assembly (GA) was composed of all participants each of whom had one representative with the authority to vote. The GA met annually during the funding period and the main tasks were to grant proper implementation of the participant's rights and obligations always in accordance with the contractual framework of the project and the Consortium Agreement and was the ultimate decision-making body of the Consortium. All other non-voting researchers working for this project joined the meetings and discussions. To facilitate the organisation and management, the scientific programme of the project was structured in work packages (WP) which together comprised the project. Each work package has been headed and coordinated by an experienced principal investigator as work package lead and a deputy leader. They were responsible for the management of their WPs. The Steering Committee (SC) shall be chaired by the Coordinator or its delegate and is comprised of the work package leaders. The SC reported and was accountable to the General Assembly and met every six months. Furthermore, monthly web or phone conferences took place to discuss the process and open tasks on a regular basis. To ensure a high standard of research and monitor the progress of the project by taking part in the annual GA Meetings a scientific advisory board was implemented.

Objectives of T-Control:

Objective 1: Development of Streptamer-based GMP compliant cell selection processes for multi-specific T cells and pT-regs

Competitive advantages of Streptamer-based cell selection processes in contrast to state of the art:

Streptamer-based selection processes benefit from the fact that the Streptamer isolation reagents can be completely removed from the isolated cells. This feature enables:

- The generation of **minimally manipulated cells products** with optimal effector function since no molecules interact with cell surface receptors or prevent their interaction with other factors
- **The Avoidance of potential side effects** from isolation reagents in the patient
- The rapid provision of cell products (within **1 day**) due to the **fast selection process** from whole (cord) blood (no *in vitro* expansion app. 20 days)

- **Serial positive selections** to be performed, targeting multiple markers with excellent recoveries and purities.
- **Fast and cost-effectively** develop primary minimally manipulated cell products due to their EMA **non-ATMP** status.
- The classification of Streptamer reagents as **ancillary products** reducing the regulatory demands for their production.

These unique competitive advantages of the Streptamer technology lead to a cost and time reduction in the development of therapeutic cell products, thus enabling the generation of the anticipated two cell products within the project duration.

Objective 2: Clinical development of the multi-specific T cell product to treat infections and tumor relapse after allogeneic HSCT

Competitive advantages of multi-specific T cell products are:

- **Optimal effector function** due to complete detachment of isolation reagents and fast cell processing
- Treatment of infection and tumor relapse post HSCT in one product
- Rapid availability of clinical cell product due to **short production time** (**1 day** and no *in vitro* expansion **of 20 days**)
- **Favorable economics** for minimally manipulated cell product due to non-ATMP status
- Multiple options for application in other indications (e.g. cancer, infections)

Using the reversible Streptamer technology the aim was to compose, in a simultaneous selection procedure, a virus pathogen-, MiHA- and TAA-specific T cell product derived from the memory and naive T cell repertoire from healthy donors to be used therapeutically or prophylactically to combat viral infections and tumor relapse after alloSCT.

Objective 3: Clinical development of the pT-reg product to treat acute GvHD after allogeneic HSCT

Competitive advantages of pT-reg products are:

- Optimal suppressive function due to complete detachment of isolation reagents and fast cell processing
- **No laborious and harmful pre-selection** (e.g. ficoll gradient) of lymphocytes needed for pTreg product selected from whole UCB
- **Unrestricted access** to UCB samples (UCB banks)
- "**Off the shelf" product** enables "industrial" manufacturing process
- Rapid availability of clinical cell product due to **short production time 1 day** (only selection, no *in vitro* expansion **app. 20 days**)
- The very **favorable economics** of the minimally manipulated cell product due to **non-ATMP status** and "off the shelf" option
- Multiple options for application in other indications (e.g. solid organ transplantation, autoimmune diseases)

Using the Streptamer technology the aim was to develop a rapid and highly reproducible method to purify pT-regs from UCB to prevent or treat severe GvHD following allogeneic HSCT.

1.3 Description of the main S&T results/foregrounds of T-Control

WP1: High purity isolation of antigen specific T-cells using combinatorial MHC Streptamer staining

Task 1: Simultaneous isolation of multi-antigen specific T cells from the memory repertoire of healthy donors (partners 3 JUNO, 1 UKW, 4 LUMC, month 1-month 12)

A standard operating procedure (SOP) was developed by partner 4 LUMC for the simultaneous isolation of virus-specific T cells directed against CMV, EBV and AdV derived antigens binding in different HLA class I molecules from the memory T cell compartment of healthy donors (**D1.1**). In this development process the performance of nanobeads and microbeads in the HLA/peptide streptamer isolations was compared (reagents supplied by partner 3 JUNO). The decision was made to use nanobeads in the ultimate protocol. The SOP was further validated by partner 1 UKW.

Task 2: Isolation of minor histocompatibility antigen (MiHA) and tumor associated antigen (TAA) specific T cells from the naïve repertoire of healthy donors; inclusion of further specificities and performance of additional isolations (partners 3 JUNO, 1 UKW, 4 LUMC, month 1-month 45)

A SOP was developed by partner 4 LUMC for the simultaneous isolation of T cells directed against the tumor associated antigens (TAA) PRAME, NY-ESO, WT-1, RHAMM and proteinase-3, and T cells against the MiHA HA-1 from HLA-A2 positive, HA-1 negative healthy donors (**D1.2**). Reagents were supplied by partner 3 JUNO and the SOP was further validated by partner 1 UKW, followed by functional testing of the isolated T cells by partner 4 LUMC.

In the project extension period partner 4 LUMC performed additional isolations using other specificities, including new HLA-types and more minor histocompatibility antigens (MiHA). Additional MiHA epitopes can now be targeted, including UTA-2/HLA-A*02:01, LRH-1/HLA-B*07:02 and LB-ARGHDIB-1R/HLA-B*07:02 (Task 2, **D1.4**). This is essential to increase the application to more patients. These MiHA are encoded by genes with a hematopoiesis restricted expression profile, allowing the induction of a specific GvL effect without coinciding GvHD. In preclinical work scientists from partner 4, LUMC, demonstrated ability to isolate MiHA-specific T cells from peripheral blood of different donors. These isolated T cells displayed differential functional avidities, including T cells capable of recognizing primary malignant cells.

Task 3: Development of a standard operational procedure (SOP) for a combined multivirus, MiHA and TAA specific T cell product (partners 3 JUNO, 1 UKW, 4 LUMC month 1-month 36)

A SOP was developed by partner 4 LUMC for the simultaneous isolation of virus-specific T cells directed against CMV, EBV and AdV derived antigens binding in different HLA class I molecules, T cells directed against the TAA PRAME, NY-ESO, WT-1, RHAMM and proteinase-3, and T cells against the MiHA HA-1 in a multi antigen specific T cell product for adoptive transfer into patients (**D1.3**). Reagents were supplied by partner 3 JUNO and the SOP was further validated by partner 1 UKW, followed by functional testing of the isolated T cells by partner 4 LUMC. Furthermore, deliverable **D1.4** *Additional SOP for newTAA or MiHA specific T cell products* has been delivered in time in month 21 (January 2015).

In the project extension period partner 4 LUMC demonstrated the ability to use this procedure for the isolation of virus-specific T cells from virus-naïve (seronegative) donors. Although the precursor frequencies of virus-specific T cells in peripheral blood of virus-naïve (seronegative) donors are extremely low, it was found to be possible to visualize these cells after repetitive enrichment steps with the HLA streptamer/nanobead technology. T cells with a diverse repertoire of functional avidities were isolated and characterized.

Task 4: Development of methods to monitor antigen specific T cells in patients treated with multi-specific T cells (Partner 3 JUNO, month 1-month 45).

- Methods were developed for optimal monitoring of the in-vivo appearance or expansion of antigen specific T cells in patients treated with multi-specific T cell products by partner 4 LUMC using fluorescently labelled peptide/HLA tetramers in multicolour flowcytometry (**D1.5**).
- In the project extension period immunological monitoring was performed for all patients that completed the follow-up period in the clinical study with multiantigen-specific T cells performed in the LUMC. In-vivo expansion of all specificities of virus-specific T cells could be demonstrated in these analyses.

In Summary: A validated SOP was developed for the GMP-grade isolation of multi antigen (CMV, AdV, EBV, TAA, MiHA) T cell products from peripheral blood of healthy donors. Using this method we demonstrated that it is possible to reproducibly isolate multi antigen specific T cell products meeting the release criteria for adoptive transfer. In the clinical trial performed by partner 4 LUMC 24 patients received a multi antigen specific T cell product so far.

By performing subsequent enrichment/purification rounds, we demonstrated that besides virus-specific memory T cells, despite their low initial frequencies TAA and MiHA specific T cells comprise an elementary component of the multi antigen specific T cell products. Moreover, we could also demonstrate the suitability of this procedure for the isolation of virus-specific T cells from peripheral blood of virus-naïve (seronegative) donors.

So far, 13 patients completed the follow-up period and were evaluated by immunological monitoring. Viral reactivations were associated with expansion of virus-specific T cells. In some patients virus-specific T cells were already present at the moment of infusion of the multiantigen-specific T cell product, whereas in other patients virus-specific T cell clearly expanded after infusion of the multiantigen-specific T cell product. In-vivo expansion of T cells directed against tumor-associated antigens (TAA) and minor histocompatibility antigens (MiHA) could not be demonstrated using direct tetramer stainings. More sensitive techniques may be required to identify potential in vivo expansion of these cells.

WP2: Isolation of clinical grade umbilical cord blood regulatory T cells for cell therapy

Summary of progress towards objectives and details for each task

The main task of WP2 was to take the selection of pT-regs from UCB, using small-scale research grade selection (<500x10^6 starting total nucleated cells (TNC)), to large-scale selection from frozen UCB units (up to 2.5x10^6 TNC). In order to be compatible with good manufacturing practice (GMP), the selection needed to be performed in an enclosed system (using clinical processing bags) and using reagents that were either clinically compliant or had a clinical grade equivalent. Single step selections of UCB pT-regs initially appeared satisfactory and, therefore, the method was optimised and a number of large-scale GMP compatible selections performed (**Tasks 1** and **2**). However, it later transpired that there were significant problems with the function of the cells immediately post isolation, requiring an amendment to the schedule to develop a two-step selection method (initial CD4+ selection followed by CD25+ selection) (**Tasks 3** and **4**). As there were also concerns regarding the final isolated cell dose, partner 2 (ANT) also explored expansion of the streptamer-selected cells using GMP compatible methods (**Tasks 5** and **6**). At the same time, our collaborators in Germany (Red Cross Blood Service Baden-Württemberg-Hesse, Frankfurt) worked on adult peripheral blood (PB) derived pT-regs as an alternative source (**Part 2**). Finally, after confirming that the streptamer selected cells could be expanded, additional experiments were performed to determine the best starting pT-reg product, the optimal expansion stimuli, and to acquire a complete phenotype and functional profile of the expanded cells for research publication (detailed **Tasks 5** and **6** and **D7.2**).

Task 1: Development of a one-step cell selection process for Streptamer-based UCB pT-reg cells (months 1-15)
Partners 2 (ANT) and 3 (JUNO) first developed a protocol for isolation of pT-regs from fresh whole UCB using an aliquot of a UCB unit (*small-scale*). The first stage of this work package was to test the robustness and consistency of this method before using frozen UCB units and higher cell numbers. Isolations were performed under research conditions (using the non-clinical IBA magnet), adjusting the amount of anti-CD25 Fab loaded onto the streptactin,

the amount of streptamer complex and the cell density. The purity of the pT-regs, defined as $CD4^+CD25^+CD127^{low}FOXP3^+$, was measured by flow cytometry (**Figure 1**). To validate the final method with fresh whole UCB, nine isolations were performed under research conditions, using 100-400 $x10^6$ TNC. The average purity of pT-regs from $CD4^+$ was 88±5% and from $CD45^+$ cells (total leukocytes) 79±7%. The average efficiency of selection (pT-reg yield compared with pT-regs present in the UCB sample) was 19±12%. This was comparable with antibody-based selections (Miltenyi Biotec) performed during the same period from cord blood mononuclear cell (CBMC) preparations (~30%).

The selection method was then optimised to select pT-regs from frozen UCB. Of note, it was necessary to add a DNase-1 treatment to the method to prevent clumps of dead/live cells forming with the streptamers. Eight isolations were performed in small-scale (starting TNC ranging from 25-400 $x10^6$ cells), with an average purity of pT-regs 88±7% of $CD4^+$ cells and 70±9% of $CD45^+$ cells, and efficiency of 12±6%. This method was then developed with UCB processing bags and the Dynal CTS magnet to a fully enclosed GMP compliant method. With the help of all partners involved in this work package, a SOP describing the isolation of pT-regs from whole UCB using CD25 streptamers was written and submitted (**D2.1**). The process was validated with three test procedures at large-scale with research grade (Buffer IS; IBA) and clinical grade (Composol / 1%human serum albumin (HSA)). A comparison of the purities at each of the stages is shown in **Figure 2**. Whilst the purity of the $CD4^+$ population remained high, the purity of $CD45^+$ cells decreased. Further analysis (not shown) showed that these were predominantly $CD3^-CD25^+$ cells and $CD7^+$ indicating, a common lymphoid progenitor. Notably, however, high-risk contaminants such as $CD3^+CD4^-$, NK and B cells were low (<20%). Based on the viability and contaminant data from all the pT-regs isolations performed using CD25 streptamers, a quality control SOP of the pT-reg cell product was established and submitted (**D2.2**). This deliverable primarily assessed pT-reg purity and viability by analysing CD45, CD3, CD4, CD127, CD25, and FOXP3 expression using flow cytometry. In the final large-scale clinical grade selection, the efficiency of selection was only 2.8%. This likely a consequence of high cell death in frozen samples compounded by a bag based (high surface area) selection.

All isolations showed good viability; around 80% with nuclear exclusion dye. In addition, the viability of pT-regs post-isolation was assessed by flow cytometry using DAPI and Annexin V at different times, up to 144 hours post-isolation. It was found that pT-reg viability was stable until 72 hours post-isolation (**Figure 3**). pT-reg viability started to decrease beyond this time and, therefore, indicates that a potential pT-reg product should be transported in less than 72hrs.

Finally, to enable the cells to be monitored in the patients post administration a flow based method utilising HLA-DR and HLA-A2 mismatches between the patient and pT-regs to identify the administered cells. This method was submitted as **D2.3**.

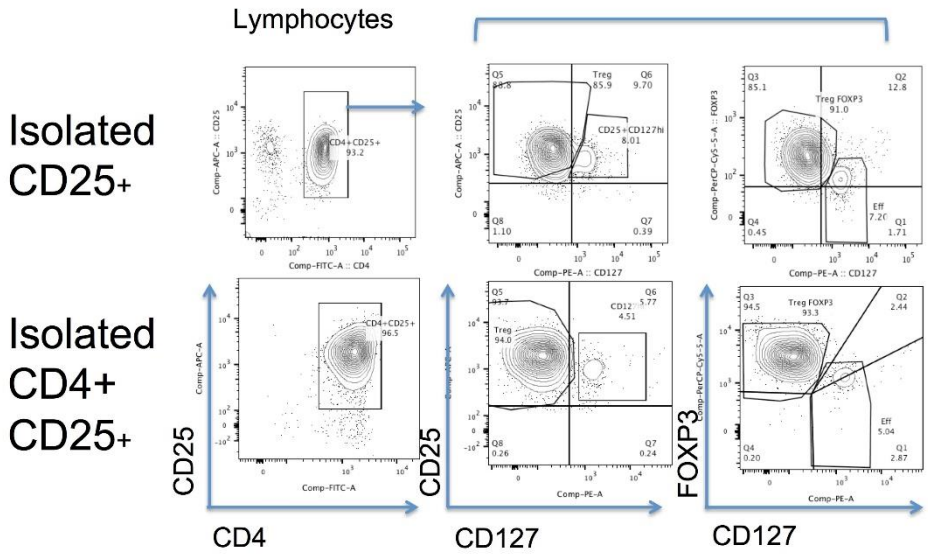


Figure 1 Characterization of streptamer isolated UCB $CD25^+$ and $CD4^+CD25^+$ cells. UCB streptamer-isolated $CD25^+$ cells (top) $CD4^+CD25^+$ cells (bottom) characterized for CD45, CD4, CD127, CD25 and FOXP3 expression. Lymphocytes by FSC and SSC were gated for CD4 and CD25 expression as shown (Left column) and then characterised for CD127 and CD25 (centre), and FOXP3 and CD127 expression (Right).

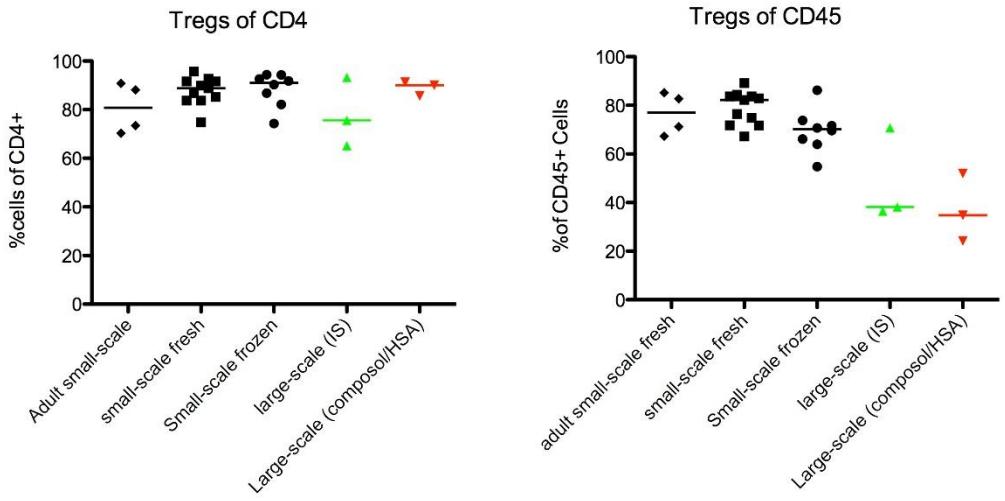


Figure 2. Treg purity from small-scale to large-scale clinical grade buffers. Shown are adult PB selections (from $CD4^+$ cells), small-scale (up $500x10^6$ TNC) selections from fresh and frozen UCB, large-scale selections (whole frozen UCB unit up to $2300x10^6$ TNC) in research grade Buffer IS (IS) or clinically compatible Composol/1% Human serum albumin (HSA). Purities are shown as proportion of $CD4^+$ cells (Left) or $CD45^+$ cells (Right).

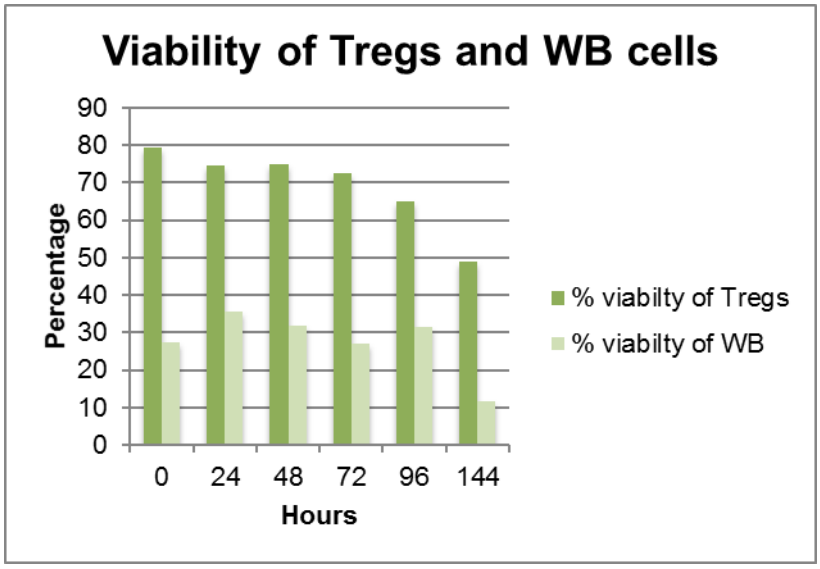


Figure 3. Treg and whole blood (lysed) viability at different times post-isolation. Post-isolation, cells were stored at 4°C in Composol/HSA at 1 x 10^6 cells/mL. Cell viability was determined by flow cytometry using Annexin V and DAPI at indicated times.

Task 2: Functional characterisation of pT-reg cell product for developing a pT-reg quality control procedure (months 3-45)

With the help of partner 1 (UKW), partner 2 (ANT) was able to assay Treg suppressive activity following a published protocol (Stemberger *et al.,* 2012, Partner 3, JUNO)[1]. In summary, this involves adult PB selected $CD4^+$ cells (Miltenyi Biotec) being labelled with CFSE to measure proliferation and then activated with OKT3 anti-CD3 in the presence and absence of the isolated pT-regs.

From this work, it was noted that the suppressive function of the $CD25^+$ streptamer selected cells from fresh whole UCB was weak when compared with pT-regs selected from either fresh adult peripheral blood (PB) or fresh isolated UCB $CD4^+$ cells (**Figure 4**). This indicated that, although the streptamer-selected cells were suppressive, isolating pT-regs from $CD4^+$ selected cells produced a more suppressive product. Furthermore, analysis of the phenotype of the selected cells using a single (CD25 only) or two-step selection method (CD25-streptamer selection from isolated

CD4+ cells) revealed a difference in the CD25 mean fluorescence intensity (MFI) of the final product; double selection yielding a higher CD25 MFI (**Figure 5**).

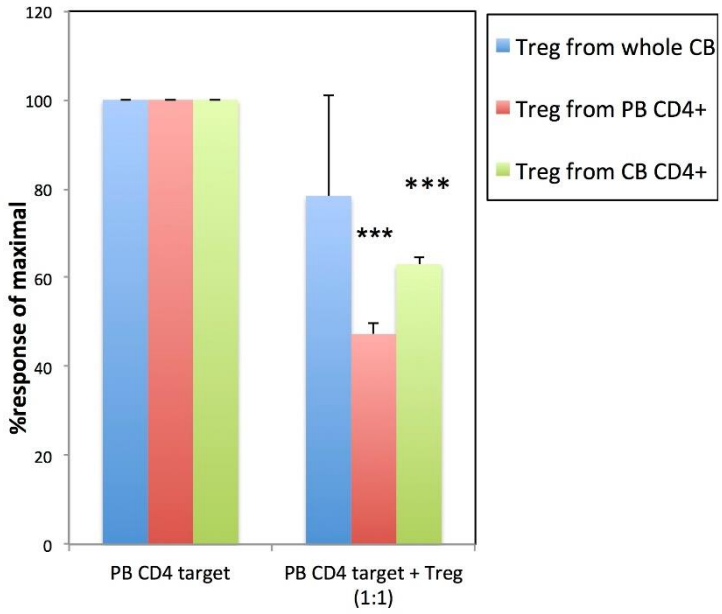


Figure 4. Proliferation of adult CD4⁺ T cells in the presence and absence of streptamer isolated pT-regs. The percentage of maximal division of CFSE labelled CD4⁺ T cells to OKT3 anti-CD3 in the absence (PB CD4 target) and in the presence (PB CD4 target + Treg) of unlabelled streptamer isolated CD25⁺ pT-regs from whole blood (blue), PB CD4⁺ cells (red) or from UCB CD4+ cells (green) at a ratio of 1:1.

Selection of UCB pT-regs using CD25⁺ streptamers produced a range of CD25 mean fluorescence intensity (MFI) in the final product (**Figure 5A**) that correlated with suppressive function (**Figure 5B;red**). In contrast, when selecting cells from adult PB, although the pT-reg products also had a range of CD25 levels, this did not impact on suppressive function (**Figure 5B; blue**). By altering the amount of anti-CD25-Fab present in the streptamer constructs, products with a range of CD25 levels could be produced which, in turn, correlated with changes in suppressive function (**Figure 5B**). The changes in CD25 MFI were not the result of contamination with CD4⁺ effector cells as the Treg purity of the CD4⁺ cells was uniformly high. Likewise, cells with high CD25 MFI did not have higher expression of FOXP3; a comparison of CD25 and FOXP3 expression is shown in (**Figure 5C)**. We, therefore, hypothesised that the streptamer selection was removing CD25 from the surface of the pT-regs and this was investigated in task 4.

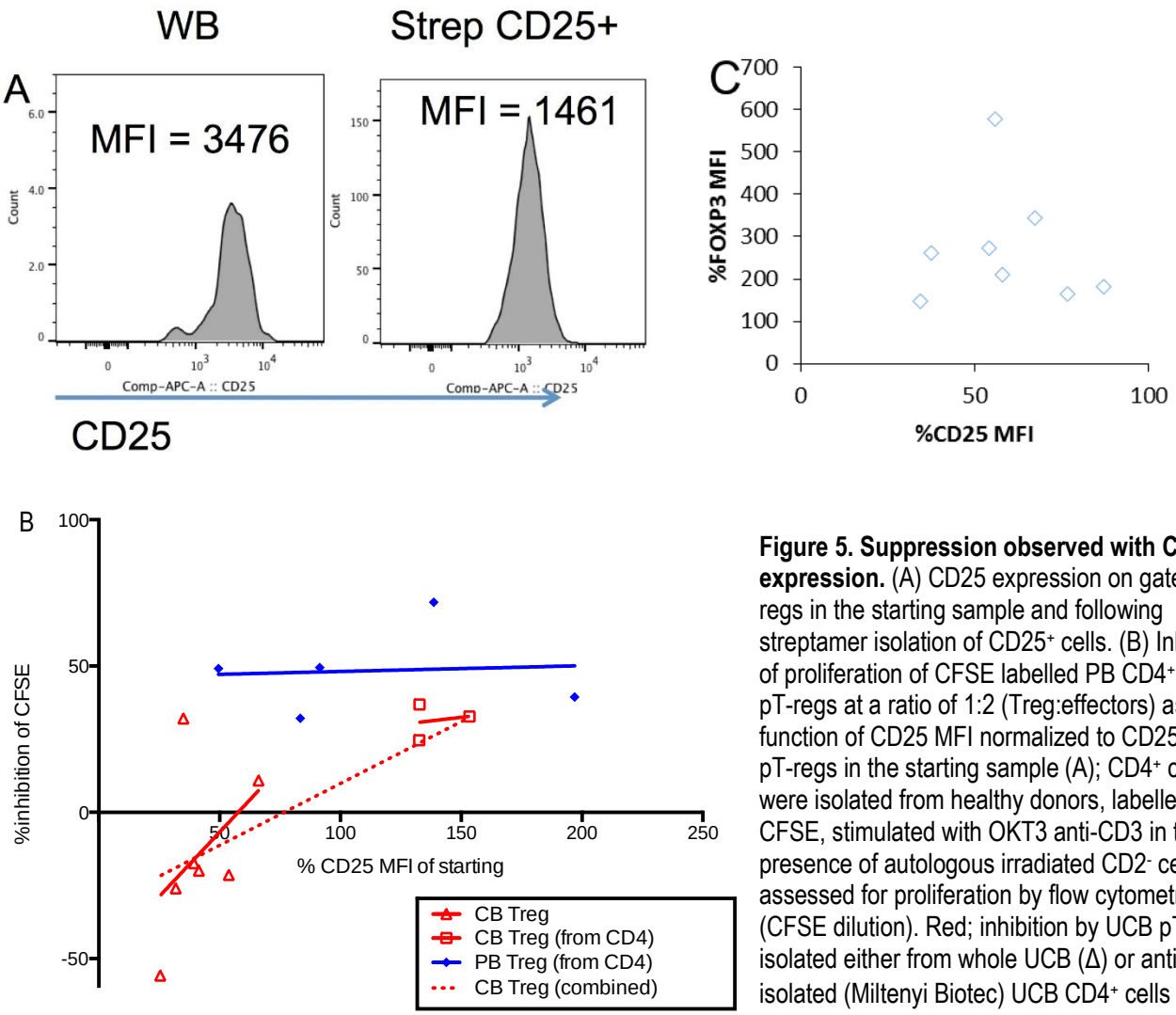


Figure 5. Suppression observed with CD25 expression. (A) CD25 expression on gated pT-regs in the starting sample and following streptamer isolation of CD25⁺ cells. (B) Inhibition of proliferation of CFSE labelled PB CD4⁺ cells by pT-regs at a ratio of 1:2 (Treg:effectors) as a function of CD25 MFI normalized to CD25 MFI of pT-regs in the starting sample (A); CD4⁺ cells were isolated from healthy donors, labelled with CFSE, stimulated with OKT3 anti-CD3 in the presence of autologous irradiated CD2⁻ cells and assessed for proliferation by flow cytometry (CFSE dilution). Red; inhibition by UCB pT-regs isolated either from whole UCB (Δ) or antibody isolated (Miltenyi Biotec) UCB CD4⁺ cells (☐). Blue; inhibition of proliferation by PB pT-regs isolated from antibody isolated PB CD4⁺ cells. Correlation %CD25 MFI and inhibition of CFSE (red dotted line, UCB Treg (combined) r^2=0.55, p=0.015. (C) FOXP3 MFI and CD25 MFI on gated CD3⁺CD4⁺CD127^{low}CD25⁺ cells.

Task 3: Development of a two-step cell selection process for Streptamer-based UCB pT-reg cells (months 18-45)
The observation that pT-regs from fresh UCB CD4⁺ cells were more suppressive lead to the development of the double streptamer selection (CD4⁺ streptamer selection followed by CD25⁺ streptamer selection). The higher CD25 MFI selection from CD4+ cells is likely due to single-step selection of CD25+ cells from whole UCB requiring more anti-CD25-Fab to be loaded onto the streptamers.

It was found that the anti-CD4-streptamer method as recommended by partner 3 (JUNO) produced a high purity CD4 product. Thus, the two-selection method was optimised by first streptamer selecting CD4⁺ cells using the recommended method and then performing a CD25⁺ streptamer selection. To assess the impact of anti-CD25-Fab loading and determine the optimal concentration when using CD4⁺ selected cells, a series of small-scale isolations were performed. CD4⁺ streptamer selections were performed from three fresh UCB (600-650x10^6 TNC) and then CD25⁺ cells selected using three streptamer constructs, each loaded with different amounts of anti-CD25 Fab (1/10, 1/20 and 1/40th of the standard dose recommended by JUNO). The average efficiency of CD4 selection was 28±8%, average pT-reg purity of CD4⁺ cells 86±8% and of CD45⁺ cells 79±8%. The three constructs produced three different ranges of CD25 MFI levels (average; 1/10= 43±12%, 1/20= 70±6% and 1/40=90%±10%). Higher CD25 MFI was at a cost of lower yield/efficiency of selection from CD4⁺ cells (1/10= 43±10%, 1/20= 27±7%, 1/40=11±4%). The same relationship between CD25 MFI and function was observed as with the single streptamer selection method; indicating that lower anti-CD25-Fab loading would likely produce the most suppressive product (**Figure 6**).

The final task was to determine the optimal anti-CD25-Fab loading dose for double selections from frozen UCB units. A series of large-scale selections in GMP compatible buffers (SSP/ 1% HSA) were performed from cryopreserved

units with different anti-CD25-Fab loading (**Table 1**). It is notable that the average purity from $CD45^+$ cells was considerably higher with the double streptamer selection (68% compared with 30% in **Figure 2**). Unfortunately, it was also clear that the increased CD25 MFI of the final product did not recover suppressive function (**Figure 6**). However, previous investigations had indicated that the cells selected from cryopreserved UCB units are able to expand to anti-CD3/28, indicating that this result is not a result of poor viability. It was, therefore, decided to determine the feasibility of expanding a functional Treg product using GMP compatible culture conditions (**Tasks 5** and **6**) and investigate the causes of the loss of function with the streptamer selected UCB CD25+ cells (See **Task 4**).

The SOP for the selection was submitted as deliverable **D2.4.** As with the single selection, quality control was assessed by CD45, CD3, CD4, CD127, CD25, and FOXP3 expression using flow cytometry. The assessment was simplified compared with deliverable **D2.2** after consultation with our GMP facility at the German Red Cross Blood Service Baden-Württemberg-Hesse, Frankfurt (Germany), to ensure that the panel could be performed on a clinical grade Canto II flow cytometer (7 colours). pT-regs were assessed as $CD45^+CD3^+CD4^+CD127^{low}CD25^+$ cells and either intranuclear fixation/permeablisation for FOXP3 expression or viability using 7AAD (7-amino-actinomycin D) exclusion staining. The assessment SOP was submitted as **D2.5.**

Table 1. Efficiency, pT-regs yield from TNC and purities for each large-scale isolation performed from frozen whole UCB with different anti-CD25-FAB loading indicated.

Isolation	Starting TNC ($x10^6$)	%CD4 viability	CD4+ ($x10^6$)	efficiency from live	%Purity of CD4+	%Purity of CD45	CD4+ CD25+ ($x10^6$)	Live Treg cell yield ($x\ 10^6$)	efficiency from CD4+	%CD25 MFI	anti-CD25-FAB loading of standard dose
1	1000	30.1	10.4	27.7	68.4	58.7	0.14	0.08	26	34	1/10
2	1300	57.7	25.4	27.2	77.7	64.8	0.52	0.34	21	58	1/20
3	1300	18.9	18.8	34.8	72.5	64.8	0.31	0.20	16	87	1/35

Task 4: Functional characterisation of pT-reg cell product isolated using the two-step method for developing a pT-reg quality control procedure (months 18-45)

As detailed in the previous task, there was a clear relationship between CD25 MFI on the final pT-reg product and suppressive function when using fresh UCB. However, when using frozen UCB, selecting CD25 MFI high cells did not restore suppressive function (**Figure 6**). As part of the extended work, we therefore investigated why UCB pT-regs isolated by anti-CD25 streptamers had low *in vitro* suppressive function. Since it was possible to select suppressive pT-regs using CD25 streptamers from fresh PB, the streptamer selection method itself did not appear to be responsible. Instead, it appeared to be related to amount of anti-CD25-Fab required when selecting from UCB. It was likely, therefore, that UCB pT-reg biology make the UBC pT-regs more susceptible to the changes in the CD25 surface levels.

We therefore tested the hypothesis that IL-2 signalling was effected by the high anti-CD25-Fab loading of the streptamers required when selecting pT-regs from whole UCB. $CD25^+$ cells were streptamer selected from fresh UCB or from PB $CD4^+$ cells (so as to compare with the functional data in **Figure 5**). Using phosphoflow, phosphorylation of STAT5 was measured in isolated cells and pT-regs in cord blood mononuclear cell (CBMC) and peripheral blood mononuclear cell (PBMC) preparations from the samples in response to incubation with IL-2 (**Figure 7**). It was clear that $CD25^+$ streptamer selected cells from UCB had a lower response to higher concentrations of IL-2 than unselected cells (**Figure 7, left**). There was also a small impairment of the STAT5 response in $CD25^+$ streptamer selected cells from adult PB (**Figure 7, right**) but the effect in the UCB was much greater (~50% effect compared with ~10%). This observation that UCB pT-regs are affected more than PB pT-regs is consistent with the loss of

function in the UCB whilst the PB pT-regs are functionally unchanged (**Figure 5**). Therefore, the effect of streptamer selection on UCB pT-reg function is the result of inherent differences in UCB and PB pT-reg biology. A single isolation from UCB was excluded as an outlier, as it had a similar CD25 MFI to the starting population and the STAT5 response was not impaired.

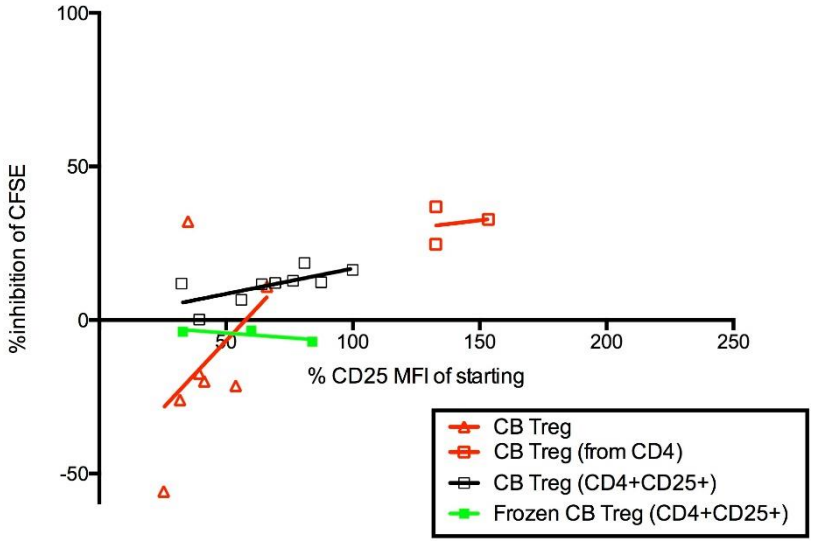
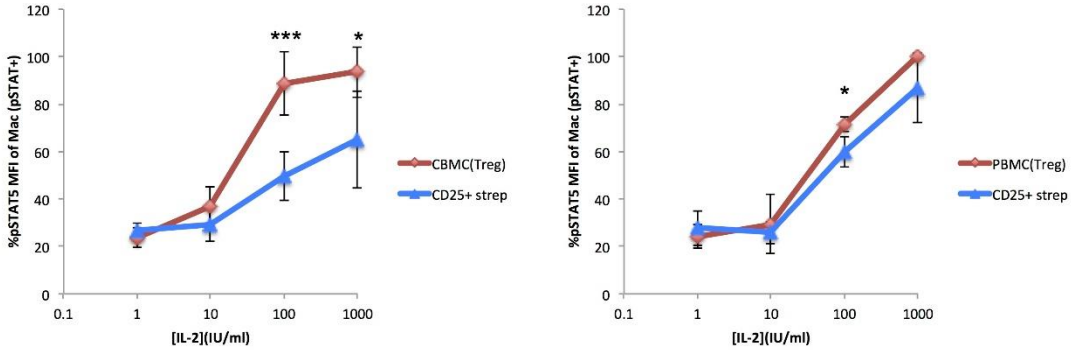


Figure 6. Suppression observed with CD25 expression on double streptamer selected cells. (A) Inhibition of proliferation of CFSE labelled PB CD4⁺ cells shown by pT-regs at a ratio of 1:2 (Treg:effectors) as a function of CD25 MFI normalized to CD25 MFI of pT-regs in the starting sample. Shown is suppressive function of (Black) inhibition by two-step CD4⁺CD25⁺ streptamer selected pT-regs from fresh UCB and (Green) inhibition by CD4⁺CD25⁺ two-step



streptamer selected pT-regs from frozen UCB superimposed onto the UCB data from **Figure 5**.
Figure 7 Response to IL-2. Shown is the response by gated pT-regs (CD4⁺CD127^{low}CD25⁺ cells) in either streptamer CD25⁺ isolated cells from UCB units (left) or from PB CD4⁺ cells (right) or unselected cells in CBMC/PBMC to IL-2 in 10 minis. The proportion of pSTAT5 MFI of labelling is normalised to the maximal response observed by gated Tregs in either CBMCs or PBMCs. Isolations were performed from three UCB units and three PB CD4⁺ samples with two selections, each of different anti-CD25-Fab loading (to generate pT-reg products with CD25 MFI lower than that of starting unselected pT-regs).

Task 5: Establish a protocol to expand UCB pT-regs (months 24-45)

Three large-scale selections were performed from frozen units using the methodology established in the previous section with a fixed 1/35 anti-CD25-FAB loading (**Table 2**).

Table 2 Efficiency, pT-regs yield from TNC and purities for each large-scale isolation performed from frozen whole UCB.

Isolation	Starting TNC (x 10^6)	CD4 viability (%)	CD4+ fraction (x 10^6)	efficiency from live CD4 (%)	Purity of CD45 (%)	Purity of CD4+ (%)	CD4+CD25+ (x 10^6)	Live Treg cell yield (x 10^6)	efficiency from CD4+ (%)
1	1376	38.5	40.2	65.8	71.4	86.8	0.43	0.31	17.4
2	1270	52.1	42.8	67.6	88.9	97.7	0.8	0.71	27.0
3	1290	45.3	21	29.9	79.0	93.6	0.14	0.11	13.7

Based upon the expansion method published by Brunstein *et al* (2011)[2], a Treg expansion protocol was developed and optimized using anti-CD3/28 Dynal beads (Dyna beads human T-activator; Life sciences) or anti-CD3/28 expansion/expander beads (Treg expansion kit; Miltenyi Biotec) as the stimulus. The products detailed in Table 1 were expanded in GMP compatible media (10% AB serum X-VIVO 15) under a range of conditions to determine the optimal method for expansion and functionality. This included use of flat or round-bottomed culture plates, addition of IL-2 (300 or 1000 IU/ml) from the beginning of the culture or day 3, and either single stimulation or including up to two additional re-stimulations. All cell lines expanded well, and all lines were suppressive when tested at one or two weeks. Ultimately, it was decided that expansion with two stimulations (re-stimulation after one week) in flat wells with 1000 IU/ml IL-2 was the preferred condition.

Further experiments were then performed to assess whether Dynal beads and Expander beads produced equivalent expanded products, and if the CD25 expression levels on the starting pT-reg product were important (as higher CD25 MFI required a sacrifice in yield). Performing a large-scale CD4 streptamer selection from frozen UCB, followed by two small-scale anti-CD25 selections (*large-small scale selection*) with different anti-CD25-Fab loading, allowed $CD4^+CD25^+$ pT-regs with different CD25 MFI to be produced. $CD4^+$ cells were streptamer selected from four frozen UCB units using the large-scale methodology and then two $CD25^+$ streptamer selections performed (small-scale) using anti-CD25 streptamer constructs loaded with either 1/15 or 1/35 doses of anti-CD25. The average CD4 selection efficiency was 24±6%, producing four pT-reg products with low CD25 MFI (59±17% with average efficiency of 19±12%) and three pT-reg products with high CD25 MFI (83±28%, efficiency 8±5%); one CD4 selection yielded insufficient cells for two $CD25^+$ selections. These isolated pT-regs were then expanded in 10% AB serum X-VIVO 15 with the addition of 1000 IU/ml IL-2 on day 0 and then every 2-3 days after, with a single re-stimulation at one week. The aim using this work was, therefore, to determine whether (a) starting CD25 MFI affected expansion; (b) the two methods of GMP expansion generated different products; (c) validate that pT-regs selected with the full large-scale (large-scale GMP compatible CD4 and CD25 selection) would expand consistently.
Our data demonstrated that, for expansion, there was no advantage of having higher CD25 MFI on the isolated cells before expansion. As shown in **Figure 8**, the cells isolated with lower amounts of anti-CD25-Fab during selection did not expand better than those with higher loading. Therefore, to validate that the isolated cells could expand after full large-scale double selection (*large-large*) and, to generate more data on the expansion with Dynal beads and Expander beads, four large-scale double selections were performed from frozen UCB units. Since, there was no advantage to having a higher CD25 MFI on the final product, the anti-CD25-streptamer was loaded with a fixed amount ($1/15^{th}$ of the IBA standard dose) of anti-CD25-FAB to maximise yield. The purity and efficiency of the selections (**Table 3**) was consistent with the previous *large-small* scale selections. All lines expanded and the data was combined with the large-small scale selection data (1/15 selected; **Figure 8**).
The expansion rate peaked at week 1 and 2 with final fold expansions of between 200-400 fold in three weeks (**Figure 8**). This is comparable to published expansion rates with UCB pT-regs and anti-CD3/28 beads[3]. This indicates that expansion of streptamer selected UCB pT-regs to produce a functionally active regulatory T cell (Treg) product is a viable ATMP. Both Dynal and Expander beads appear to have similar growth characteristics over a three week culture period. All cells were suppressive (see **Task 6, Figure 11**) and there were only small variations in the phenotype of the expanded Tregs (**Task 6, Figures 9 and 10**) with the two bead systems. The expansion SOP was submitted as deliverable **D2.6**.

Isolation	Starting TNC (x 10^6)	CD4 viability (% in thawed unit)	CD4+ fraction (x 10^6)	efficiency from live CD4 (%)	Purity of CD45 (%)	Purity of CD4+ (%)	CD4+CD25+ (x 10^6)	Live pT-reg cell yield (x 10^6)	efficiency from CD4+ (%)	CD25MFI
1	1400	39.8	101	51.1	47.7	87.2	1.51	0.72	9.5	38.1
2	1400	38.6	53.68	38.5	87.5	91.7	0.72	0.63	17.1	52.0

3	1200	44.7	34.4	42.2	85.7	89.9	0.23	0.20	9.6	84.3
4	1300	67.9	62.6	33.8	86.3	91.0	0.79	0.68	12.1	72.5

Table 3 Efficiency, pT-regs yield from TNC and purities for each large isolation performed from frozen whole UCB. A large-scale CD4 selection was performed followed by a large-scale selection using 1/15 loaded anti-CD25 streptamers.

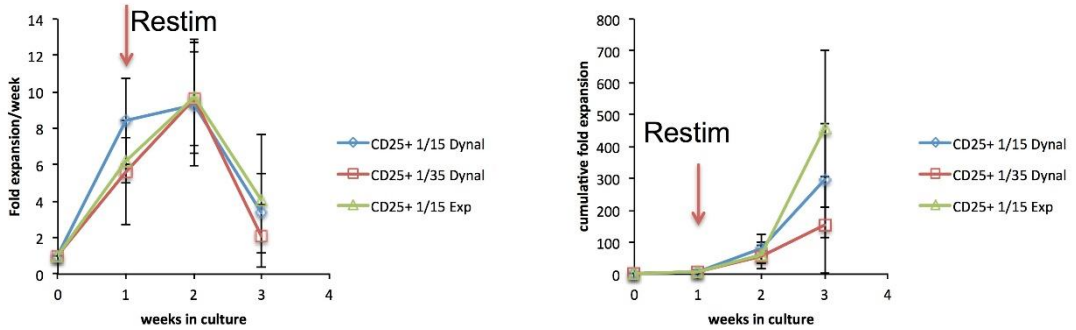
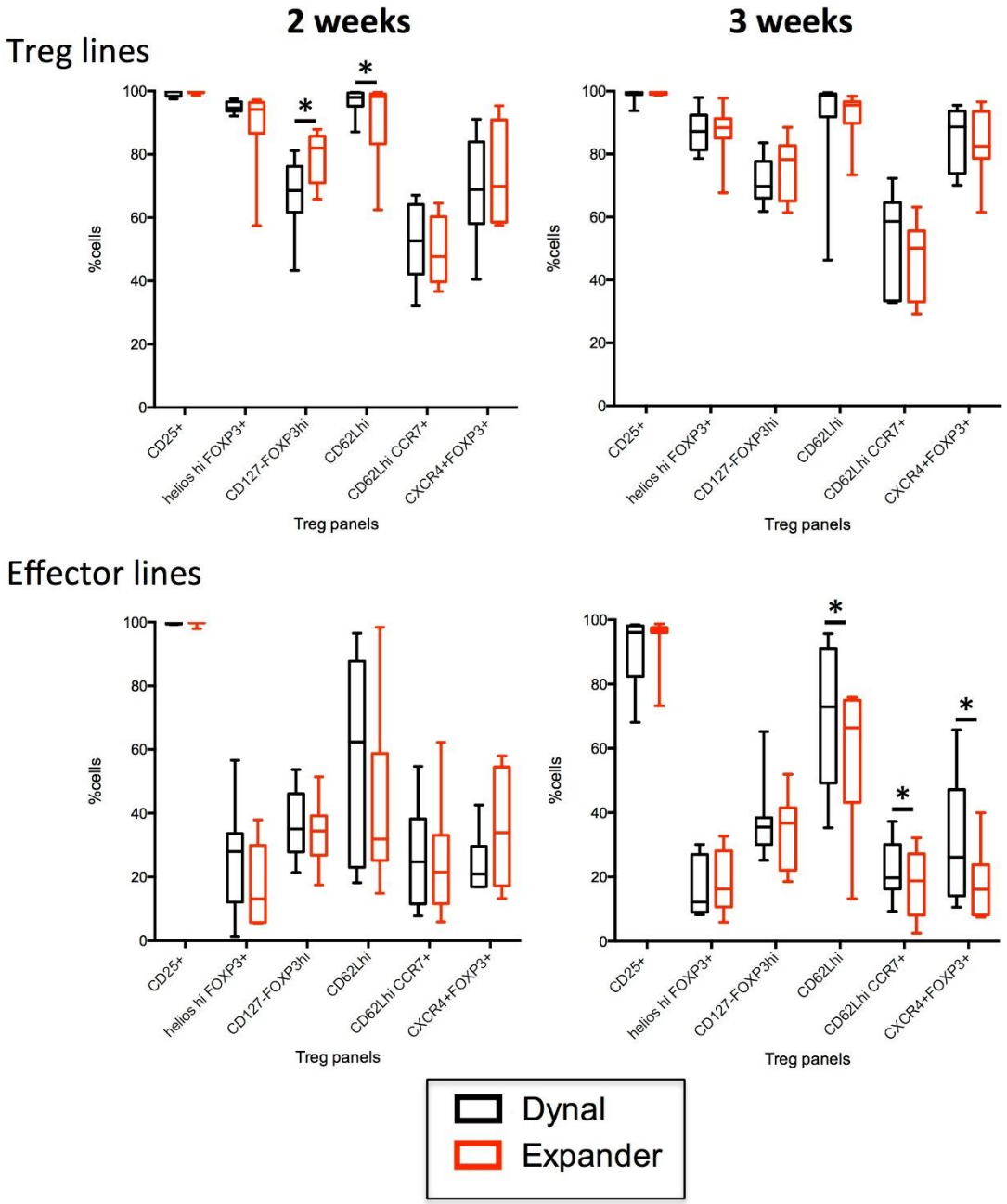


Figure 8 Growth rate of streptamer selected pT-regs to Dynal or Expander anti-CD3/28 beads and 1000 IU/ml IL-2. (Left) fold expansion observed each week in culture, (Right) cumulative fold expansion. pT-regs were selected from large-scale streptamer selected CD4 cells (from frozen) using either 1/15 (small-scale n=4, large-scale n=4) or 1/35 (small scale n=3) loading of anti-CD25-fab onto the streptamers. Isolated cells were expanded with either Dynal (n=8 for 1/15 and n=3 for 1/35) or Expander (n=7) anti-CD3/28 beads as indicated.

Task 6: Phenotypic and functional characterisation of expanded UCB Tregs (months 24-45)
Significant results
During the expansion, $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ effectors (the negative fraction from the $CD25^+$ streptamer selection) were followed for Treg exhaustion, Treg function and chemokine receptor markers by flow cytometry. This was to determine if the Treg populations were stable (maintain a Treg phenotype compared to the effectors whilst showing low sign of exhaustion). Since there was no advantage to selecting a high CD25 MFI population, the data from the large-small scale selections (1/15 anti-CD25-Fab; four lines) and the large-scale selections (1/15 anti-CD25-Fab; four lines) were combined to compare Tregs expanded to Dynal and Expander beads with effectors expanded in parallel. **Figure 9** shows the cell lines characterised for Treg markers.
The Treg lines were significantly different for the majority of markers when compared to their control effector lines, apart from CD25 expression (high in all lines). The Treg lines retained high proportions of both $Helios^{hi}FOXP3^+$ cells, and $CD127^{low}FOXP3^{hi}$ cells; the activated Treg phenotype. In addition, Tregs maintained a $CD62L^+$ phenotype (entry into lymph nodes) and a central memory/naïve phenotype with high proportions of $CD62L^+CCR7^+$ cells. Finally, the UCB Tregs are $CXCR4^+$ (bone marrow homing), which was also stably expressed at three weeks (**Figure 9**). A comparison of Dynal and Expander bead stimulated Tregs did show significant differences in the activated Treg ($CD127^-FOXP3^{hi}$) phenotype and CD62L expression, but these differences were not significant at week 3 (**Figure 9**). As these cells are intended for cell therapy it was important to determine if the cell lines acquired any markers of T cell exhaustion, namely high expression of 2B4, and PD-1[4]. Whilst there was some expression of these markers the proportions were relatively low at week three (**Figure 10A** and **B**). Two lines expanded very poorly (cell death was evident) and these stood out as outliers (**Figure 10B**) with high and sustained PD-1 expression. CXCR3 expression can be associated with a stable memory phenotype (with loss in the exhausted cells)[4]. However, only small amounts were present at late time points, even in the effector lines (not shown), and a subsequent cytokine receptor screen (See **Figure 10G**) indicates that the Tregs favoured a Th2-like ($CXCR3^-$ phenotype). The UCB Tregs retain roughly equal proportions of $alpha4^+beta7^+$ and $alpha4^+beta7^-$ cells after three weeks (**Figure 10C** and **D**); our experience has been that this gastrointestinal tract homing marker is present to high levels in UCB pT-regs. The expander beads (**Figure 10, red**) produced cells with a slightly lower median $alpha4^+beta7^+$ population, which might indicate that the two beads are not exactly equivalent.
Recently, Brunstein *et al*. (2016), expanded UCB Tregs with a transgenic K562 cell line generating a 10,000 fold expansion of clinical grade UCB Tregs[5]. They demonstrated that after the expansion period, the $CD4^+CD25^+CD127^{low}Helios^+FOXP3^+$ (full Treg phenotype) population were on average (median) 45% $CD62L^+$, 5% $OX40^+$ and 1-2% $41BB^+$. OX40 and 41BB can be expressed on Tregs but they are associated with down regulation of Treg function. Performing a similar characterisation of our own Treg lines indicates that after three weeks of

culture, we have low numbers of OX40$^+$ cells with the Dynal beads and a median of 10% positive with the Expander beads (**Figure 10E** and **F**). The proportion of 41BB$^+$ cells was <10% for both Dynal bead and Expander beads, respectively (**Figure 10E** and **F**). We do, however, have more CD62L expression (>90%, **Figure 10E** and **F**). This could suggest that our lines have retained a more central memory phenotype. However, it should be noted that the Brunstein *et al.* expansion had a 10,000 fold expansion compared with our 200-400 fold expansion.

Finally, Brunstein *et al*. (2016) described that their expanded Treg lines had a predominately Th2-like chemokine profile, with low levels of Th1, Th17 and virtually no Th22-like cells being present[5]. An analysis of our lines for the same markers indicates that both Dynal and Expander cultured Treg lines have median 60-70% Th2-like phenotype,



10% Th1-like, 20% Th17 and very low levels of Th22-like cells (**Figure 10G**).

Figure 9 Phenotype of Treg and effector lines for Treg markers. Shown is the proportion of the subsets shown on CD45$^+$CD3$^+$CD4$^+$ live (eFloura 506 dye negative) gated cells in either Dynal (Black) or Expander (red) anti-CD3/28 bead expanded cells after 2 or 3 weeks of culture with 1000 IU/ml IL-2. Shown is Box and Whiskers with Wilcoxon paired T-test (* = $p < 0.05$).

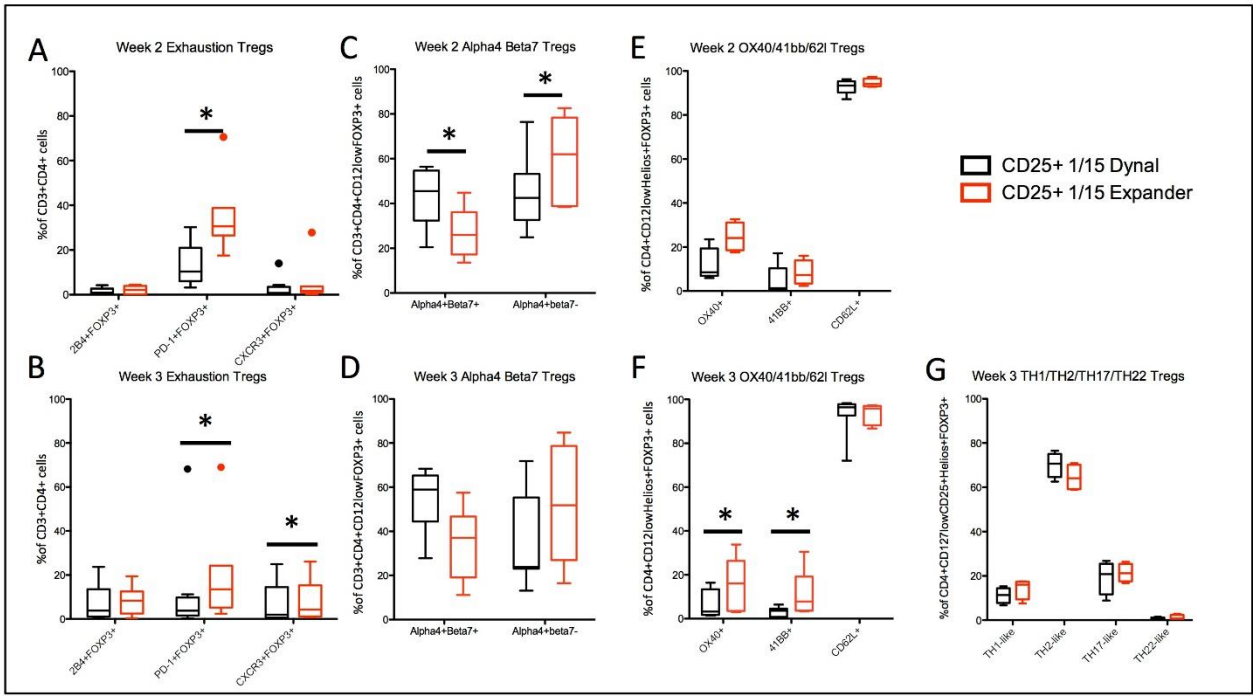


Figure 10 Phenotype of Treg lines at week 2 (upper) and week 3 (lower) of culture. Exhaustion markers (A and B); live CD3$^+$CD4$^+$ gated cells for 2B4$^+$FOXP3$^+$, PD-1$^+$FOXP3$^+$ and CXCR3$^+$FOXP3$^+$ cells. Alpha4 Beta7 (C and D); live CD3$^+$CD4$^+$CD127^{low}FOXP3$^+$ gated cells for alpha 4(CD49d) and Integrin Beta7. Live OX40/41BB/CD62L (E and F); CD4$^+$CD127^{low}Helios$^+$FOXP3$^+$ gated cells for proportion of OX40, 41BB and CD62L$^+$ cells. TH1/TH2/TH17/TH22 like (G); live CD4$^+$CD127^{low}CD25$^+$Helios$^+$FOXP3$^+$ gated cells for proportions of CXCR3$^+$ (TH1-like), CXCR3$^-$CCR6$^-$ (TH2-like), CXCR3$^-$CCR4$^+$CCR6$^+$CCR10$^-$ (TH17-like) and CXCR3$^-$CCR4$^+$CCR6$^+$CCR10$^+$ (TH22-like). Shown is the proportion of the subsets shown on gated cells in either Dynal (Black) or expander (red) anti-CD3/28 bead expanded cells after 2 or 3 weeks of culture with 1000 IU/ml IL-2. Shown is Box and Whiskers with Tukey exclusion of outliers (shown as single points for A and B). *p <0.05 by Wilcoxon paired T-test.

Figure 11 shows that Treg lines expanded with Dynal or Expander beads are significantly more suppressive than the control effector lines (red compared with black). At high ratios of effector line to CFSE labelled CD4 target cells inhibition of proliferation is observed. However, this titrates away such that at 1:4, the majority of effector lines augment proliferation (negative inhibition). This effect is likely due competition for resources at high cell numbers whilst at low cell numbers the cell line becomes a source of pro-inflammatory cytokines (since they are primed cells). It is, therefore, important to show that the Treg lines have greater levels of suppression and are still suppressing at a ratio of 1:4 Tregs to CD4 target cells. There seems to be no major difference in the suppressive function of the Tregs based on the expansion method.

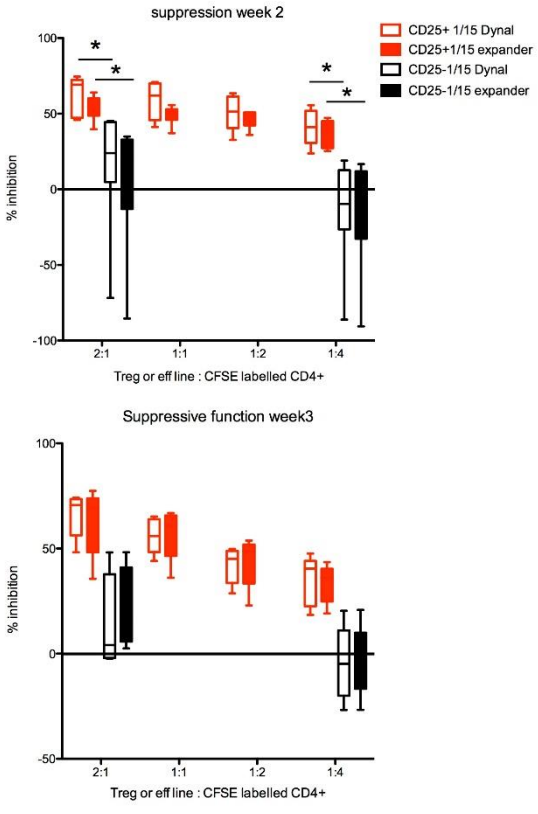


Figure 11. Suppression observed with expanded Tregs or effectors. Inhibition of proliferation of CFSE labelled PB CD4$^+$ cells to soluble anti-CD3 (OKT3) shown by CD25$^+$ derived Tregs (red) or CD25- derived effectors (black) expanded from the anti-CD25 loading, with the method indicated and at the ratios indicated. * p <0.05 by Wilcoxon paired T-test.

In conclusion, this indicates that there are a few phenotypic differences between expanding with Dynal or Expander

anti-CD3/28 beads but the Treg lines retain Treg markers, have low levels of exhaustion markers and are largely in agreement with previously published UCB expanded lines (isolated using the CliniMACS system) that have been utilized in clinical trials. Also, despite low functional activity immediately post isolation, the Treg lines quickly become highly suppressive.

Part 2: Summary of progress concerning pT-regs from adult peripheral blood - German Red Cross Blood Service Baden-Württemberg-Hesse, Frankfurt

Preliminary data by the Munich group (Stemberger, C *et al.)*[1] with small-scale isolations using the Streptamer-Fab technology had demonstrated that, in principle, isolation of primary regulatory T cells (pT-regs or Tregs) can be achieved with adequate purity by sequential selection of CD4+, CD25+ and CD45RA cells. However, extremely low recoveries questioned clinical applicability because of potential inability to isolate clinically relevant target cell doses. In contrast to UCB-Treg, where a Treg dose of 5K/kg was targeted, the clinical investigators requested a thousand-fold higher clinical dose for adult-blood derived Tregs, because work of other groups had indicated formidable expansion potential of UCB-derived, but not adult blood-derived Treg. While in principle cell doses up to 10^{10} can be routinely extracted by steady-state apheresis, a prohibitively expensive process would be needed because of the necessity to perform at least the initial CD4 selection step, in parallel, on several magnets, using multiples of reagents, tubing sets, magnetic extractors and labour.

Concerning the Treg isolations from adult peripheral blood apheresis products, we therefore started to scale the isolation procedure up to a starting material of $1x10^9$ WBCs from a donor lymphocyte preparation by simply multiplying the reagent quantities used in the small-scale pilot experiments. Since with that technology we were not able to reach Treg purities (see **Table 1** and **Figure 1**) in the end product that the clinical group deemed clinically suitable, we tried to optimize Fab/Streptamer bead conjugation times and relative quantities thereof. Additionally, we unsuccessfully tried to improve the isolation protocol by first isolating CD25+ cells followed by a CD4+ isolation step (data not shown) or by adding a third (CD45RA) isolation step after CD4 and CD25 enrichment (Run 10). Unfortunately, albeit possibly in retrospect not entirely surprisingly given that CD25 expression is not restricted to Tregs, we were never able to reach the minimal Treg (flow cytometrically defined as CD4+CD25+CD127- FoxP3+) purity of >75% of T-cells in the end product pre-defined as the target by the clinical group. Furthermore, due to deplorable target cell recoveries, we fell significantly short of generating Treg doses which met the clinical group's pre-defined targets for a clinical application.

Table 4: Results from Treg isolations from adult peripheral blood samples with the two-step strategy (serial CD4/CD25 enrichment). Starting material: $1x10^9$ WBCs from steady-state leukoapheresis preparations from voluntary donors. *Run 10 was performed as three-step isolation (serial CD4/CD25/CD45RA enrichment) starting with $5x10^9$ WBCs.

Run	viable in $CD45^+$ [%]	Treg in $CD3^+CD4^+$ [%]	Treg in viable $CD45^+$ [%]	Total amount of Treg in EP [$x10^6$]	Treg recovery compared to starting material [%]
1	66.1	88.0	55.7	0.8	1.5
2	98.2	56.1	55.0	3.7	5.7
3	98.6	52.0	51.5	1.1	1.5
4	97.1	65.6	64.4	0.4	1.2
5	98.2	57.1	50.3	0.9	2.4
6	96.3	70.4	66.1	0.3	16.7

7	99.1	43.1	42.2	1.7	2.0
8	99.7	19.6	19.2	3.5	7.0
9	99.7	33.9	33.1	5.1	16.5
10*	97.3	23.6	22.8	0.8	0.5

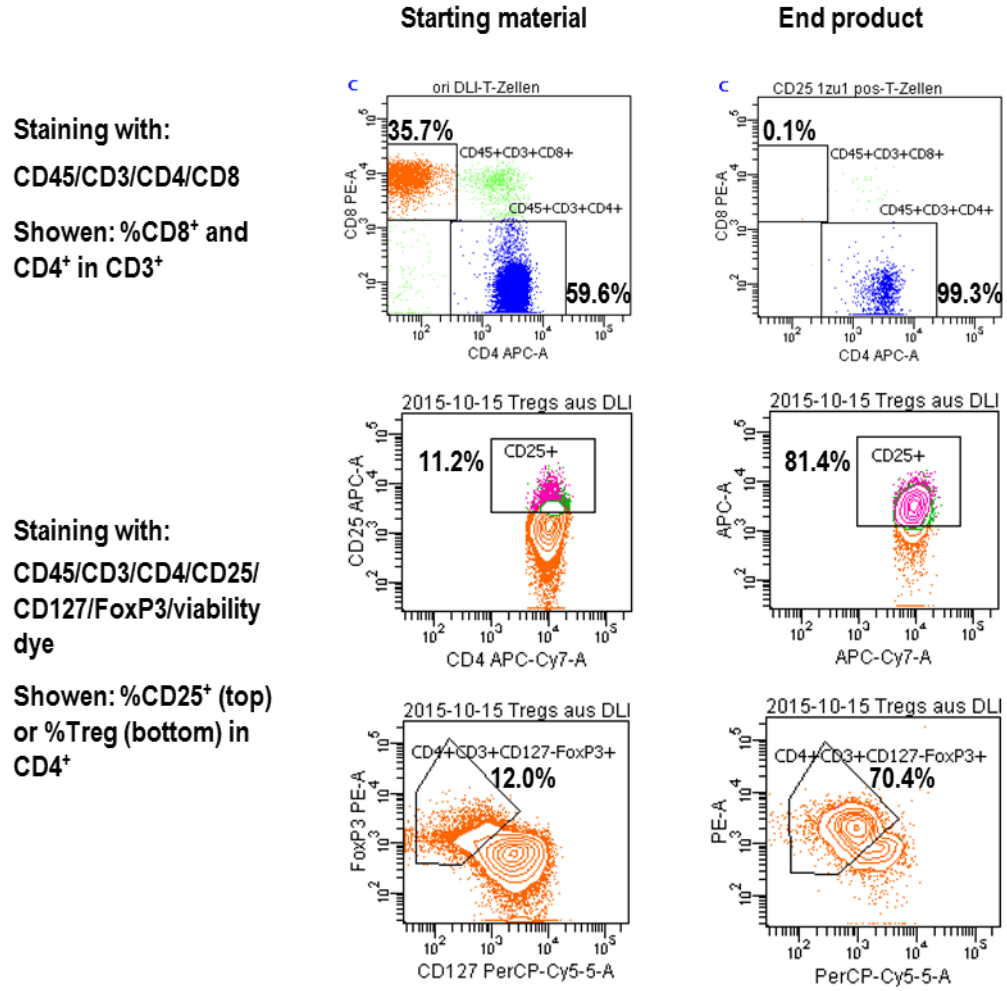


Figure 12 Exemplary results (run 6) from flow cytometric quality control measurements shown

Concluding remarks

In conclusion we have demonstrated that streptamers can be used to isolate, to high purity, pT-regs from frozen UCB units in a GMP compliant manner, in line with the original remit of the FP7 proposal. The original question was to test the feasibility of using unmanipulated pT-reg and we have determined that function and cell dose would be more practical with an expanded product. Thus in furtherance, and incompliance with the amendments, we have demonstrated that streptamer selected pT-regs can be expanded to equivalent levels to antibody selected cells, and maintain Treg function. Potential clinical avenues are full GMP expansion, pre-clinical validation and clinical trial. Further biological studies could include microarray analysis of UCB Tregs, cytokine suppression, and cytokine release profile. Material was collected throughout the project to enable these possible research questions to be explored.

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cord blood: safety profile and detection kinetics. *Blood* **117,** 1061–70 (2011).
3. McKenna, D. H. *et al.* Optimization of cGMP purification and expansion of umbilical cord blood-derived T-regulatory cells in support of first-in-human clinical trials. *Cytotherapy* (2016). doi:10.1016/j.jcyt.2016.10.011
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WP3: Clinical trial for the treatment of infections and tumor relapse after allogeneic HSCT

Viral infections and disease relapse are major complications in the interval between T cell depleted allogeneic stem cell transplantation (TCD alloSCT) and donor lymphocyte infusion (DLI). In this phase I/II study the feasibility and safety of the generation and preemptive administration of donor T cells targeting CMV, EBV and AdV to restore viral immunity together with T cells targeting tumor associated antigens (TAA) and minor histocompatibility antigens (MiHA) to boost the graft versus leukemia (GVL) response is evaluated. Efficacy is assessed by in-vivo appearance/expansions of target Ag-specific T cells in the peripheral blood detected with direct tetramer staining, and the effect on viral reactivation and disease relapse until DLI was given 20 weeks later. HLA-A*02^{+} patients treated for a hematological malignancy with an HLA-matched TCD alloSCT from a CMV^{+} and/or EBV^{+} donor were included. 6-8 weeks after alloSCT, T cells directed against HLA-A*02-restricted peptides of CMV, EBV and AdV, and the TAA NY-ESO-1, WT-1, RHAMM, PRAME and proteinase 3 were isolated under GMP conditions in 1 day, using the reversible streptamer-nanobead technology by cliniMACS selection out of the naïve and memory T cell compartment from 2*10^9 donor PBMC. Depending on the patient/donor HLA-typing, additional streptamers targeting viral peptides in HLA-A*01, A*24, B*07, or B*08 were added to the procedure as well as the HLA-A*02/HA-1h streptamer in case of MiHA disparity in the GVL direction.

Results:

1. Feasibility:

In the 2 trials in WP3 up to now 35 patients were included (planned patient number 34 patients).

In the LUMC in Leiden, the Netherlands, 27 patients were enrolled in the clinical trial. For these patients, 27 multi Ag-specific T cell products were generated of which 26 met the release criteria, containing 9-18 different specificities per product.The products consisted of 0.4-26.5*10^6 cells containing purities of 46,0-99.9% target Ag-specific CD8+ T cells within the T cell compartment. In all products CMV and/or EBV as well as AdV virus-specific memory T cells were isolated comprising 99% of the target Ag-specific CD8+ T cells, while the other 1% included the TAA and MiHA specificities and/or CMV specific T cells from seronegative donors. The isolation of multi-antigen-specific T cells in Frankfurt (for the Würzburg patients) was similarly successful – purity of > 50% target Antigen-specific T cells and numbers of > 1 x 10(6) Ag-specific T cells were determined for the multi-antigen-specific T cell products.

None of the products contained numbers of non-specific T cells exceeding the maximal dose allowed to be transferred to the patients: 24 of the 26 products were infused. For 2 patients the product was successfully generated, but administration to the patient was cancelled due to progressive GvHD immediately prior to the day of planned infusion, making these patients not anymore eligible for treatment. These data show that the treatment is feasible: the product could be generated successfully in more than 95% of the cases, and 89% of the patients included in the study received the product. In Würzburg where a strict CD34+ selection is performed prior to the administration of multi-antigen specific T cells in 3 patients multiantigen-specific T cells could not be administrated due to insufficient in vitro T cell depletion prior to allografting thus requiring post-transplant immunosuppression. For the ethical committee of Würzburg University only allowed to recruit elderly patients (> 60 years) or patients with significant comorbidities (Sorror score > 3) patients at high risk for transplant-related mortality were included. Thus one patient died after successful engraftment due to a ischemic heart disease (myocardial infarction) before receiving the multi-antigen-specific T cell product. The death was clearly not related to the transplantation procedure. None of

the patients developed severe acute GvHD (of any grade) following CD34+-selected peripheral blood stem cell transplantation.

2. Safety:

In total 30 patients received a multi-antigen-specific T cell product up to now – with several more to be transfused in the next weeks.. 24/27 patients received a streptamer-selected multiantigen-specific T cell product after in vivo T cell depletion using Campath in the bag as a part of the T cell depletion procedure. None of the patients experienced significant transfusion related toxicity. No patients show signs of increase of GVHD with 13 patients having fully completed the follow-up period thus far. In 5 patients the complete follow-up period until regular DLI at 6 months after transplantation could not be reached, but this was not due to toxicity of the product: early death (n=1, nephrotic syndrome), chemotherapy for relapse (n=2), early application of unmodified DLI because of mixed chimerism in a patient with high risk disease (n=1), and treatment with rituximab for an EBV-PTLD (n=1). 6 patients are still in active follow-up. These data show in these patients that the treatment with streptamer-selected multiantigen-specific T cell product is safe, since no infusion related toxicity occurred and no GVHD developed. Similarly following transplantation of CD34 selected peripheral blood stem cell grafts no acute GvHD occurred – and in addition none of the patients who received a multi-antigen-specific T cell product after allografting of CD34-selected peripheral blood stem cells developed acute or chronic GvHD:

3. Efficacy

A total of at least 34 patients will be studied for efficacy data, and thus far 16 patients completed the follow-up period and 13 were evaluated by immunological and viral monitoring. None of the patients experienced AdV reactivations, and in at least 1 patient AdV-specific T cells appeared after infusion indicating active control of the virus. Of 9 patients at risks, in 6 patients CMV reactivations were observed. In all 6, CMV-specific T cells were detected and CMV was cleared. No CMV disease developed. Three patients experienced an EBV reactivation. In 2 patients, the virus was cleared without profound expansion of EBV-specific T cells, but 1 patient required treatment for an EBV-PTLD indicating failure of the treatment although ultimately EBV-specific T cells appeared and the virus was cleared. In some patients virus-specific T cells were already present at the moment of infusion of the multiantigen-specific T cell product, whereas in other patients virus-specific T cell clearly expanded only after infusion of the multiantigen-specific T cell product. None of the patients who received streptamer-selected multi-antigen specific T cells following a CD34 selected peripheral blood stem cell graft showed virus reactivations or viral disease. Thus far, in-vivo expansion of T cells directed against tumor-associated antigens (TAA) and minor histocompatibility antigens (MiHA) could not be demonstrated using direct tetramer stainings. But only two patients showed a relapse of their malignancy that required chemotherapy treatment prior to the scheduled DLI at six months following transplant. In summary, in 90% of the evaluable patients at this stage of follow-up prevention or control of viral reactivation could be achieved. The strategy thus allowed a significant reduction of acute GvHD without an increase in viral reactivations or disease. Longer follow-up which will be performed according to the requirement of the ethicals committees will allow to assess the graft-versus leukemia/tumor efficacy when compared to a T- replete allograft (historical data).

WP5: Development and production of Streptamer reagents and corresponding GMP cell products

Task 1: Development and production of multi-specific Streptamer products (month 01-12)

The first step of the development of the multi-specific Streptamer products was the definition of the individual Streptamers necessary for a comprehensive set of reagents for the composition of multi-specific Streptamer products. Partner 04 LUMC and we (Partner 03 Stage) therefore determined the different alleles and antigens which had to be produced. The selection of alleles and antigens was driven by the completeness of the coverage of the patient population. Due to the HLA polymorphism of the population the selected set of alleles will cover between 80 and 90% of the population. Product coverage is very important for the recruitment of patients in the clinical trial and the benefit of the product for a large patient population.

After selection of the reagents (see Table 5) every single Streptamer reagent was cloned, expressed, purified and tested for function (milestone M5.1). The functional testing was again done by LUMC who have cell lines available necessary for testing the various different alleles and antigens.

The different Streptamers consist of three different molecules, the alpha chain, the beta microglobulin and the corresponding peptide antigen. The first two proteins are recombinantly produced in bacteria whereas the peptide is purchased from a vendor in the required quality. After production of the starting materials proteins are refolded with the peptide to yield the respective Streptamer. Unfortunately this refolding process is rather ineffective yielding only 5% product from the respective amounts of starting materials.

In addition to the functional testing by LUMC, we defined biochemical QC release criteria comprising size exclusion chromatography of the refolded Streptamer molecule and mass spectrometry for the identification of the respective Streptamer product (**Figure 13**).

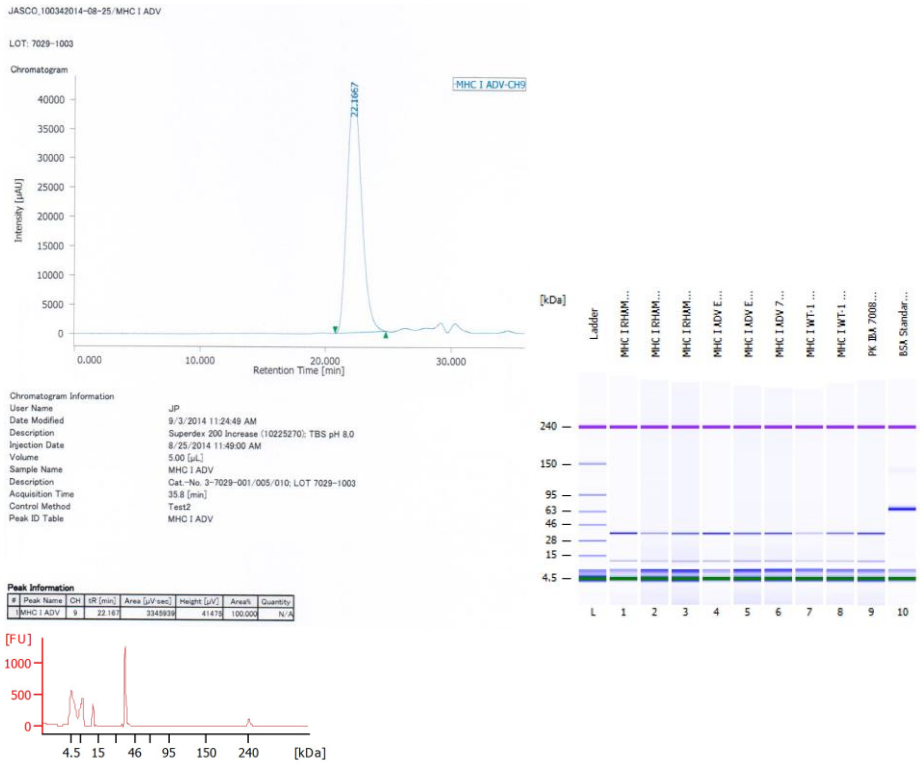

Figure 3 Quality control of the refolded MHC complexes. The left figure represents and HPLC elution profile from a refolded MHC I ADV. The figure shows the very high purity of the MHC's (>99%). The purity of other MHC complexes is at least 95%. The middle and right figure shows a electropherogram of different MHC complexes (middle) and one the elution profile of one MHC complex (right). In agreement with stage could see profile the complexes show very high purity (>99%).

The production process of the Streptamer reagents is controlled by a quality management system ensuring the reproducible quality of the delivered reagents. Every MHC Streptamer complex is released with a certificate of analysis demonstrating the main parameters of the product (**Figure 14**).

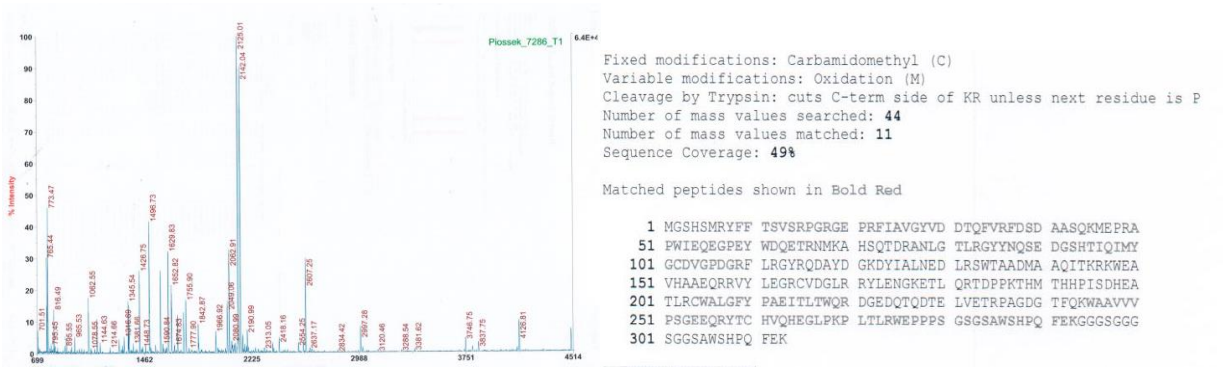

Figure 4 Quality control of the refolded MHC complexes. The left figure represents a mass spectrum from a refolded MHC I ADV. The figure shows the sequence coverage of about 50% of the MHC's. Thus the identity of the MHC complex is determined by this analysis.

We have developed a production process yielding clinically applicable reagents (see details in deliverable D5.1). This means that during the production procedure certain requirements raised by the regulatory agencies from Germany and UK have to be fulfilled. Final filling of the reagents has to be in the GMP environment to prevent non-sterile products. Furthermore the production process should prevent the contamination of TSE and endotoxins of the final product. These regulations have a significant reach through to the processes which have to be performed in a very controlled manner.

Extensive discussions among the project partners have led to the list of MHC Streptamer depicted below. According to the concept of multi-specific T-cells virus specific MHC Streptamers and tumour associated MHC Streptamers have been identified for the selection of the corresponding T-cells.

Primary Tregs (pTregs) are characterized by several markers among them the surface markers CD4 and CD25 which are used for pTreg selection from blood or other starting materials (e.g. PBMC´s). Together with our colleagues from Antony Nolan London (Partner 02 AN) we have developed CD4 and CD25 Fab-Streptamer reagents which can be fully dissociated from the selected pTreg cell population (milestone M5.2). Development of the reagents comprises the cloning of the Fabs from corresponding Hybridoma clones, the mutagenesis of the cloned Fabs to engineer off rates enabling their complete dissociation and their fermentation in E.coli to avoid mammalian cell culture with its different risk profile (see details in deliverable D5.2). The resulting Fab-Streptamer reagents were tested by AN for their ability to select pTregs from cord blood. The reagents proved to be fully reversible and capable to select suppressive pTregs from cord blood. Similar to the multi-specific T cells the Streptamers for pTreg isolation were released due to a strict QC protocol including electropherography and mass spectrometry. These data are summarized in a certificate of analysis as main release document for the reagents (**Figure 15**).

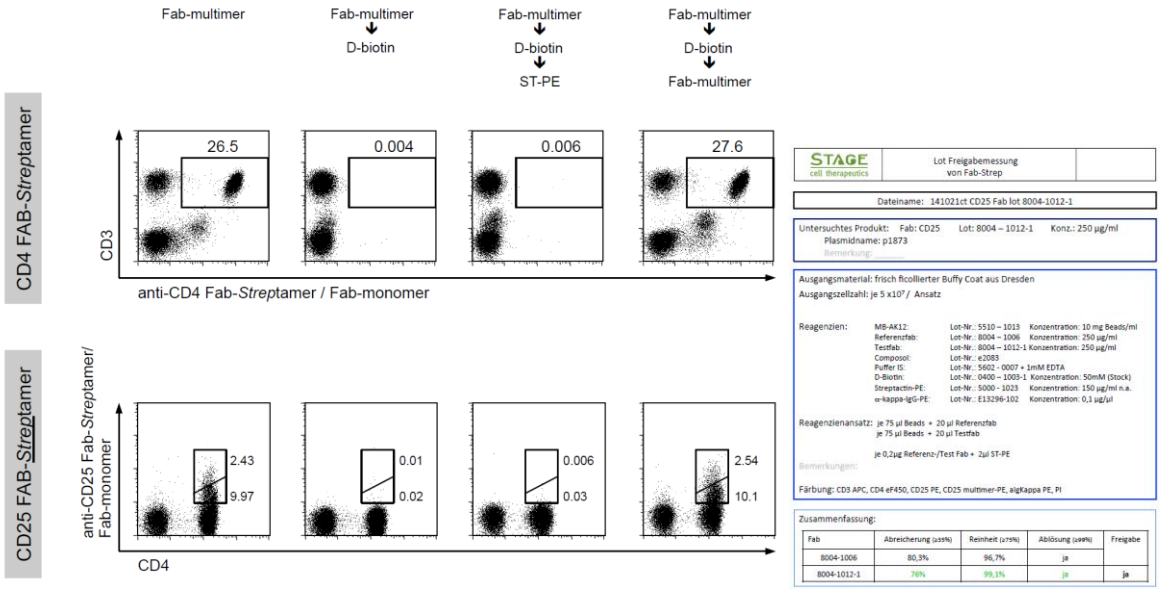

Figure 15 FACS analysis of a CD4 CD25 serial enrichment using Streptamer reagents (right) and the corresponding release document for a CD 25 Fab reagent batch.

Task 3: Development of a Streptamer-based, GMP compliant cell selection process for multispecific T cell product and the pT-reg cell product (month 6 – month 24)

As described within the last periodic report, discussions among the project partners have led to the list of MHC-Streptamer depicted below. According to the concept of multi-specific T-cells, virus-specific MHC-Streptamers as well as tumour associated MHC-Streptamers have been identified for the combined selection of the corresponding T-cells.

	Allel/Antigen HLA-				
Origin	A1	A2	A24	B7	B8
CMV	pp50 VTEHDTLLY	pp65 NLVPMVATV	pp65 QYDPVAALF	pp65 TERVTGGGM	IE-1 QIKVRVDMV
EBV		BMLF-1 GLCTLVAML	LMP2 PYLFWLAAI	EBNA-3A RPPIFIRRL	BZLF-1 RAKFKQLL
ADV	TDLGQNLLY	E1a LLDQLIEEV	Hexon TYFSLNNKF	Hexon KPYSGTAYNAL	
MiHA - Ha-1		VLHDDLLEA			
MiHA – UTA2-1		QLLNSVLTL			
MiHA – LB-ARGHDIB-1R				LPRACWREA	
MiHA – LRH1				TPNQRQNVC	
TAA 1 - WT-1		RMFPNAPYL			
TAA 2 - NY-ESO 1		SLLMWITQV			
TAA 3 – Rhamm		ILSLELMKL			
TAA 4 - Prame 1		VLDGLDVLL			

CD4 Fab Strep	CD25 Fab Strep	CD45RA Fab Strep	

Microbeads	Nanobeads		

Table 5: List of delivered MHC-Strep molecules, Fab-Strep molecules, Micro- and Nanobeads

Clinical reagent sets for the above mentioned reagents were continuously manufactured and provided to the partners to supply the currently running (LUMC) or started clinical trials (UKW/Fra). All MHC-I- and Nanobead reagents (see table) were produced and formulated for a total of $1x10^9$ starting cells. Due to the modular design, the reagents can be assembled according to the needs of the patient in regard to total cell numbers, allele- and antigen composition. In addition, Microbead based clinical selection kits comprised of magnetic Microbeads (size range of about 1.5-2µm) and Fab Strep reagents (see table 1 above) for purification of pTregs were continued for single as well as serial selection processes (also allowing modular adaptation to actual selection needs; single Fab- and Microbead aliquots for selection out of $1x10^9$ cells) to further enable in-house process development in pTreg selection.

In close interaction with the partners Leiden University Medical Center (LUMC) and Blutspendedienst Baden-Württemberg-Hessen (as manufacturing entity) together with the Universitätsklinikum Würzburg (as study/transplantation center), GMP compliant selection processes were developed that allow for selection of multi-specific T cells from donor apheresis materials.
In detail this comprises a combined selection of the individual cell populations by one-step magnetic cell selection using MHC-I Streptamer-functionalized Nanobeads (size range of about 50-70nm; selection on CliniMACS).
Together with the colleagues at LUMC in Leiden we succeeded to design a selection process by combined multiple MHC-I Streptamer Nanobeads (up to 8 different MHC-Streptamer specificities according to table 1) with excellent performance.

For this process (manufacturing permission and approval for the study design was obtained by the Dutch authorities end of 2014) we provided 12 reagent kits consisting of 32 vials Nanobeads (for $5x10^9$ cells), 133 vials MHC products (for $1x10^9$ cells) and 12 vials D-biotin solution over the last reporting period for the manufacturing of further cell products that could all be released and allowed treatment of patients and completion of the clinical trial.
The method/process described above was also successfully transferred and used to establish manufacturing of a highly comparable cell product (up to 7 different MHC-Streptamer specificities (restricted to HLA-A0201 only) according to table 1) at the Blutspendedienst in Frankfurt for use in WP3 (clinical trial at UKW, treatment of infections and tumor relapse after allo-HSCT). Manufacturing permission and acceptance of the study protocol was obtained from the PEI in March 2016. To date we manufactured and provided approx. 50 reagent kits to the partners and the fmulti-specific cell products were generated and released in Frankfurt in December 2016. In one case although being derived from a CMV-seronegative donor, comparable to the selection results in Leiden the selected cells showed high purity and could be successfully transplanted.

Instead of the failed and discontinued selection of unsuppressive low-yield cryo-pTregs, we continued to develop a two- or three-step selection process (CD4 -> CD25 -> CD45RA) of Tregs from fresh adult apheresis- or blood products by use of Fab-Streptamer Microbeads. Here pTregs are selected by repeated magnetic selections on a DynaMag CTS with single species of surface-marker specific beads followed by entire removal of all reagents prior to the succeeding step. Besides the process developmental efforts reported earlier (work on single or double selection of pTregs from relatively low-cell containing Buffy Coats in close collaboration with project partner Blutspendedienst Baden-Württemberg-Hessen) we continued to perform additional runs on larger starting cell numbers more closely reflecting actual clinical situations. We performed 3 selections – 2 double (CD4 -> CD25) and one triple selection (CD4 -> CD25 -> CD45RA) from whole buffy coats and a leukapheresis product covering about $0.5\text{-}1x10^9$ cells. Besides showing the technical feasibility (all steps can be performed in a closed GMP compliant process using clinical/GMP manufacturing compliant buffers, bags and tubing sets that is also able to handle the larger cell numbers and volumes) good purities and yields could be obtained directly after isolation. If short term cultured the obtained cells (triple selected pTregs from donor apheresis starting material) proliferated well and all cells showed a clear CD4+CD25+FoxP3+CD127+ Treg phenotype. In addition, based on a comparable selection process using the selection equipment the according process-related SOPs could be generated that would allow for a rapid transition of the process into validation and clinical application.

Task 4: Production of clinical Streptamer reagent sets as well as the corresponding multispecific T cell product for the anticipated clinical trials (month 7 – month 45)
Clinical reagents sets (MHC-Streptamer reagents including Nanobeads) are already produced for the multi-specific T cell products/trial in Leiden (LUMC) as already described in the last report. The newly developed format was also provided after tech transfer for the recently started second multi-specifc T cell trial in UKW/Frankfurt. In total we provided 13 clinical MHC-Streptamer kits with 21 different specificities that allowed manufacturing cell products and subsequent treatment of patients within two clinical trials.

In addition, we also produced clinical grade Fab-Streptamer reagents (reactive for human CD4, CD25 and CD45RA including Microbeads) for the further process development on the clinical multi-parameter selection of pTregs from human blood sources at large scale. These single Fab- and Microbead aliquots were produced for selection out of $1x10^9$ cells for several developmental selections.

1.4 The potential impact

Socio-economic impact and the wider societal implications of the project, contribution to Community and social objectives

The consortium aimed to improve the general outcome of HSCT and to develop strategies to reduce HSCT-related complications, thus leading to cost-savings for hospitals and for the EU. With the transfer of a multi antigen specific T-cell product developed in WP1 after a T-cell depleted HSCT in the clinical trial (WP3) the project could show in already 33 patients treated that the incidence of a GvHD could be significantly reduced. By reducing the incidence of acute and chronic graft versus host disease but preventing infectious complications - the two most common complications of allogeneic stem cell transplantation - this could significantly reduce morbidity and mortality for patients undergoing allogeneic stem cell transplantation as well as reduce the main cost drivers for HSCT.

The societal impact of this project is to improve the outcome of allogeneic HSCT thanks to a treatment using selected cellular products derived from T-cell subsets from the donor. The aim is to expand the number of patients who will benefit from immune cell-based therapies based on (a) an effective immune response against tumor cells, (b) providing protective immunity against opportunistic infections, (c) improving transplant procedures to reduce its risks and to optimize its benefits by the use of subsequent immunotherapy and (d) creating interactions between European populations through donor registries and typing centers. The resultant social benefits will be achieved, in the long-term, via an increased cure of diseases with a high incidence and a consequent decrease in treatment costs and losses, because of a reduction in productivity. T-Control has generated knowledge that is likely to generate commercially valuable intellectual property that may create employment through facilitation of European enterprises in the area of health care. T-Control made a valuable contribution for an improvement in the health care for EU citizens and the development of new technology based pharmaceuticals that can be exploited worldwide.

The cell products have the potential to address other indications with currently incurable diseases. For example the multi-specific T-cell product is planned to be tested in the treatment of solid tumors (melanoma, renal cell carcinoma, bronchial carcinoma, triple negative breast cancer, etc.). Currently, the mostly inefficient treatments for these indications are associated with severe side effects. In contrast to cell therapeutics, conventional drugs are dosed in short intervals; cell medications are dosed in long intervals if not once with significantly less side effects. European patients will benefit from these therapeutic means by gaining a better overall survival and a better quality of life.

Spectacular advances of cell therapy of solid tumors such as melanoma have been reported. In this rapidly evolving field the T-control products can make a significant medical and economic contribution, esp. the multiantigen-specific T-cell product is an attractive cell product to be used to treat residual disease and/or viral infections in patients undergoing solid organ or stem cell transplantation as well as patients with haematological or solid tumors following intensive chemotherapy. JUNO will license out certain indications to interested pharma partners that can quickly develop and commercialize the products. The entrance of big pharma companies in the field of cell therapy will boost the development of the anticipated cell products in important indications and their economic impact in Europe and worldwide

The production method of the multi-functional T-cell with the Streptamers is not yet published, but already for a mono-specific product. The innovation height to use instead of one MHC-Streptamer complex several different in a very similar protocol is due to our patent attorney not sufficient for new intellectual property. Even if there would have been a small chance to get IP, T-control had to consider if the economic value of such a patent justifies the investment especially in the light that the Streptamer technology is patented world-wide and thus fully protects both the multi-specific product and the pTreg product.

Patent application for a cell product or an isolation procedure

JUNO has had extensive discussions with its patent attorney whether the multiantigen-specific T cell product or the pTreg product or the cell production procedures for their production could be patented.
Because the product generated from cord blood was not functional from cord blood cells, was found not to suppress effector T cells the decision by JUNO was not to file a patent for the cord blood-derived Tregs generated according to the protocol developed in T Control. In contrast Tregs selected/generated from adult blood showed functional characteristics of regulatory T cells. This procedure will thus be checked for its novelty and patentability.

The multiantigen-specific T cell product is manufactured with the delivered reagents from our colleagues in Leiden and Frankfurt/Würzburg very reproducibly with high yield and high purity. This was prerequisite for the clinical use of these products in more than 30 patients so far. The technology transfer offices from Leiden and Würzburg currently evaluate if the clinical data are patentable. If so the consortium will apply for a patent protecting the clinical data of the multiantigenspecific T cell product.
With respect to the patentability of the selection technology our patent attorney argued that multiantigen-specific T cells as such has been published before with a much less complex product but the principle was disclosed. The production method with the Streptamers is not yet published for a multiantigen-specific product but for a monospecific product. The innovation height to use instead of one MHC-Streptamer complex several different in a very similar protocol is due to our patent attorney not sufficient for new intellectual property. Even if there would have been a small chance to get IP we have to consider if the economical value of such a patent justifies the investment especially in the light that the Streptamer technology is patented world wide and fully protects the multiantigen-specific product and the pTreg product. Especially since the FDA has increased the meaning of the cell production process and has indicated the change of the process is a change of the product. This dramatically increases the impact of proprietary cell processing technology because the protection of the process would imply the protection of the product.
In summary we can conclude that we have decided to apply a conservative patent strategy to the T control findings partly because of the degree of novelty of the results and partly because of the substantial product protection via the Streptamer patent portfolio.

Main dissemination activities and exploitation of results

The T-control consortium had a broad dissemination and exploitation strategy to reach out to different stakeholder groups like the scientific community (especially via publications), physicians (via network activities with other sites) and towards patients and public (project flyer, website and factsheet)

Publications:

- Isolation of clinical grade cord blood regulatory T-cells using reversible Streptamer technology, R. Duggleby,*, D. Shah, R. Laza, C. Stemberger, S. Gomez, L. Germeroth, S. Mielke, H. Bonig, K. Latham, H. Einsele, S. Querol, A. Madrigal, A. Saudemont, P395 – Bone Marrow Transplantation Volume 50, Issue S1 (March 2015)
- Isolation and function of clinical grade cord blood regulatory T-cells using reversible Streptamer technology Richard C. Duggleby* , Divya Shah, Raquel Laza, Christian Stemberger, Susana Gomez, Lothar Germeroth, Andrea Jochheim-Richter, Stephan Mielke, Halvard Bönig, Katy Latham, Hermann Einsele, Sergio Querol, Alejandro Madrigal1, Aurore Saudemont, P388 – Bone Marrow Transplantation Volume 51, Issue S1 (March 2016)

Planned Publications

- Use of reversible Streptamer technology to isolate clinical grade regulatory T-cells from frozen umbilical cord blood units for cell therapy; Richard Duggleby, Divya K. Shah, Raquel Laza-Briviesca, Carmen Salguero Aranda, Christian Stemberger, Susana Gomez, Lothar Germeroth, Andrea Jochheim-Richter, Stephan Mielke, Halvard, Boenig, Katy Latham, Hermann Einsele, Sergio Querol, Robert Danby, Alejandro Madrigal, Aurore Saudemont; A162- Bone Marrow Transplantation (in press) (March 2017)
- Characterization of Tumor Associated Antigen and Minor Histocompatibility Antigen Specific T-cells Derived from Healthy Donors for Cellular Immunotherapy Strategies, M.C.J. Roex, L. Hageman, L. Germeroth, C.J.M. Halkes, J.H.F. Falkenburg, and I. Jedema
- Simultaneous Isolation of High and Low Frequency T-cell Specificities from Healthy Donors Using the Streptamer-Nanobead Isolation Technique, M.C.J. Roex, L. Hageman, E. van Liempt, L. Germeroth, C.J.M. Halkes, J.H.F. Falkenburg, and I. Jedema

- Multi-Antigen Specific T-cells for Prevention of Viral Infections after T-cell Depleted Allogeneic Stem Cell Transplantation – a Phase I/II study, M.C.J. Roex, P. van Balen, L. Hageman, H.M. van Egmond, S.A.J. Veld, C. Hoogstraten, E. van Liempt, L. Germeroth, H. Einsele, C.J.M. Halkes, I. Jedema, and J.H.F. Falkenburg

Visits of specialised cell therapy centers

In the period September 2016 – March 2017 Prof. Falkenburg visited all university medical centers in the Netherlands to present T-Control results and to discuss the future of adoptive cell therapy in the Netherlands. Our recent results, including the results from the multiantigen-specific T-cell study performed within the T-Control consortium, illustrate that application of virus-specific T-cells in a pre-emptive rather than a pure prophylactic setting can be a (cost)effective strategy to support viral control in immune compromised patients. During his visits to the different centers Prof. Falkenburg discussed the transplantation strategies in the different centers, the incidence of viral complications after transplantation and the methods and frequency of monitoring of these complications, and the current activities in the development of new transplantation/post transplantation immunotherapy strategies that are being pursued in the different centers. This is an important step towards harmonization of treatment in the Netherlands.
The results of T-Control were presented to patients and their relatives at two informative meetings in June and November 2016. Invitations for these meetings was not restricted to patients that were enrolled in the T-Control trial, but was open for all patients and relatives that were interested in allogeneic stem cell transplantation and immune therapy.
Results of the clinical trial on adoptive transfer of multiantigen-specific T-cell products performed in the LUMC are presented at several scientific meetings. The audience included specialists from all cell therapy centers world-wide.

The UKW team visited several centers in Germany (Regensburg, Erlangen, Munich, Hannover, Kiel, Freiburg, Bad Homburg) to present the T-Control study. The cell selection strategy, the GMP-process of T cell selection, the analysis of the cell composition especially of virus-, MiHA, and TAA-specific T cells, their detection and their reconstitution was discussed with the centers.

In addition a close collaboration in stem cell selection with visits in Würzburg but also at the collaborating centers in Milano, Pavia, Rome, Barcelona, Navarra, Sevilla and Prague, Brno, Poznan and Cluji was initiated. These centers have started using our technology of T cell selection.

Topic	Location	Date	Audience (students, post docs, laboratory staff?)	Number reached
Allogeneic stem cell transplantation and immune therapy	University Medical Centers in NL	Sept.2016 – March 2017	Staff	7 university medical centers
Allogeneic stem cell transplantation and immune therapy	LUMC, Leiden	June 2016 and Nov 2016	Patients and relatives	500
Various scientific conferences, including ASH, EBMT, EHA and national meetings. Results from T Control clinical trials with multiantigen-specific T-cells (see presentations overview)	Different in NL, Europe and USA	2015-2017	Scientists, physicians	>1000
Tregs und Multispecifics	Berlin-Brandenburg center for regenerative medicines (BRCT)	28.09.2015	Staff	
Multispecifics	Universitätsklinikum Dresden	30.09.2015	Scientists	
Tregs und Multispecifics	Technische Universität	13.04.2016	Scientists	

	München, TUMcells			
Tregs und Multispecifics	Blutspendedienst Ost, Dresden	30.11.2015	Physicians, Scientists	

Communication

- The T-Control **website** was designed by partner 5 GABO:mi and launched on October 31st 2013. The T-Control website (www.t-control.info) informs the public about the project, its objectives and partners as well as of its dissemination activities and has been updated regularly. The project website has been visited by 18,104 visitors since project start (excluding visits below 30 sec to ensure no bots are counted). Beside the homepage of the project website the main sites of interest are the news section, general information on the project and the partner's institutions, especially of the recruiting sites. Besides English speaking visitors (48%) the site has been visited mainly by German (30%), Italian (6%) and Dutch (5%) users.
- A T-Control **project flyer** has also been produced by GABO:mi and disseminated during various conferences and meetings.
- A T-Control **illustration** has been designed by GABO:mi together with a scientific illustrator explaining the main objectives of T-Control at a glance. The illustration has been designed as simple as possible to make the information available also for the general public without scientific background. The illustration was published on the project website and was available for any other dissemination activities.
- The project **fact sheet** has been developed to give all partners press department easy access to publishable data and key facts. The fact sheet may also be used at various dissemination and exploitation activities and meetings.

Outlook and future research

The data obtained from the trials in WP3 up to now – we will still do the longer follow up according to the requirements of the ethics committee - clearly show that the strategy of T cell depletion and add back of multiantigen-specific T cells after 6 – 8 weeks significantly reduced the rate of acute and chronic GvHD when compared to a T-replete allograft. In addition the data generated up to now also demonstrate that the add back of pathogen-specific T cells seems to allow more rapid reconstitution of pathogen-specfic T cells post-transplant as indicated by the lack of an increased rate of infectious complications when compared to after a T-replete allograft.
Longer follow up to analyze the relapse rates and additional analyses of the frequency of TAA- and MiHA-specific T cells in the peripheral blood at later time points will be performed. These data will then allow to assess whether the late add-back of donor T cells directed against TAAs and MiHAgs after a T cell-depleted allograft will sufficiently control residual disease following alloSCT and thus prevent an increased relapse rate.
Similar studies with other strategies to deplete alloreactive T cells and to boost anti-pathogen and anti-tumor immune responses post-alloSCT are currently explored also at other institutions (CD45RA depletion, CD34 selection and add-back of central memory T cells). Our strong belief is that allogeneic stem cell transplantation will only remain a cornerstone of the treatment of patients with high risk haematological diseases if transplant-related morbidity and mortality can be reduced – but not at the expense of an increased rate of infectious complications or an increased risk of relapses of the underlying malignancy.
Thus T-control showed a novel strategy to improve allogeneic stem cell transplantation, which will allow to move the procedure also to patients with advanced autoimmune disorders, elderly patients or patients with co-morbidities by reducing significantly acute and chronic GvHD and to avoid the potential toxicities of post-transplant immunosuppression. Thus, this strategy will allow to extend allografting to a much larger patient population which could not be offered this curative treatment strategy up to now (due to very high risk of complications). This novel strategy will also allow to evaluate allografting for patients
In addition we have demonstrated that streptamers can be used to isolate, to high purity, pT-regs from frozen UCB units in a GMP compliant manner, in line with the original remit of the FP7 proposal. The original question was to test the feasibility of using unmanipulated pT-regs and we have determined that function and cell dose would be more practical with an expanded product. In further experiments we have demonstrated that streptamer selected pT-regs can be expanded to equivalent levels to antibody selected cells, and maintain Treg function. Potential clinical

avenues are full GMP expansion, pre-clinical validation and clinical trial. Further biological studies could include microarray analyses of UCB Tregs, cytokine suppression, and cytokine release profile. Material was collected throughout the project to enable these possible research questions to be explored. The plan for the future is to use the expanded pT-regs for a pilot trial for patients steroid-refractory chronic GvHD. For optimal expansion and activation of isolated and transfused pT-regs we will use low dose IL-2.

Section 2 – Use and dissemination of foreground

Please see ECAS.

Section 3 – Report on societal implications

Please see ECAS.