Adoptive T cell therapy is a promising treatment strategy for patients with different types of cancer. The methods used for generation of high numbers of tumor specific T cells usually require long-term *ex vivo* culture, which frequently lead to generation of terminally differentiated effector cells, demonstrating low persistence *in vivo*. Therefore, optimization of protocols for generation of T cells for adoptive cell therapy is warranted. The aim of this work was to develop a protocol for expansion of antigen-specific T cells using Dynabeads CD3/CD28 to obtain T cells expressing markers important for *in vivo* persistence and survival. To achieve high numbers of antigen-specific T cells following expansion, we have tested the effect of depleting regulatory T cells using Dynabeads CD25 and including a pre-stimulation step with peptide prior to the non-specific expansion with Dynabeads. Our data demonstrate that virus- and tumor specific T cells can be expanded to high numbers using Dynabeads CD3/CD28 following optimization of the culture conditions. The expansion protocol presented here results in enrichment of antigen-specific CD8⁺ T cells with an early/intermediate memory phenotype. This is observed even when the antigen-specific CD8⁺ T cells demonstrated a terminal effector phenotype prior to expansion. This protocol thus results in expanded T cells with a phenotypic profile which may increase the chance of retaining long-term persistence following adoptive transfer. Based on these data we have developed a cGMP protocol for expansion of tumor specific T cells for adoptive T cell therapy.

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1. Introduction

Adoptive T cell therapy for malignant diseases is defined as the infusion of T lymphocytes with the objective of eradicating tumor cells and prevent relapse. Reports from early clinical trials have shown that T cell therapy is a promising strategy for cancer treatment and demonstrate the feasibility and safety of this immunotherapeutic intervention (Rosenberg et al., 2004; Dudley and Rosenberg, 2003). The impressive clinical result of using *ex vivo* expanded tumor infiltrating lymphocytes in patients with advanced malignant melanoma has encouraged many groups to investigate the possibility of using adoptively transferred T cells for treating other types of cancer (Dudley et al., 2002a).

To obtain adequate numbers of T cells for infusion, T cells need to be cultured and expanded *ex vivo*. Many of the currently used methods are laborious and time-consuming (>45 days) and require large numbers of feeder cells which
may limit their clinical application (Yee et al., 2000; Dudley et al., 2002b). Moreover, some of these methods generate a final T cell product dominated by terminally differentiated T cells with a predisposition towards activation induced cell death, skewing of the T cell repertoire, and poor in vivo survival (Powell, Jr, et al., 2006). In a previous study (Huang et al., 2005), long-term persistent T cells were found to express high levels of CD28 and CD27, compared to short-term persistent T cells. Moreover, the long-term persistent T cells demonstrated low expression of the terminally differentiation marker CD57. T cell expansion protocols favoring this phenotype are therefore required.

High numbers of regulatory T cells (Tregs) are found in late stage cancer patients, both in the tumor and systemically. The presence of Tregs may thus negatively impact both the expansion of specific T cells ex vivo, and the subsequent efficacy of adoptively transferred T cells (Antony et al., 2005). Removing Tregs prior to ex vivo expansion may accordingly improve both the final yield of antigen (Ag) specific T cells after expansion and avoid possible inhibitory effects of transferring Tregs into the patient.

The frequency of tumor specific T cells in peripheral blood is generally low. This may limit the efficacy of adoptively transferred T cells expanded from peripheral blood by non-selective activation protocols. Inclusion of an Ag-specific pre-stimulation step prior to non-specific stimulation/expansion has been shown to improve the absolute number of specific T cells in the final product by increasing the frequency of such cells in the starting population (Dang et al., 2007).

Dynabeads coated with antibodies against CD3 and CD28 (Dynabeads CD3/CD28) are designed to mimic the interaction between T cells and APCs in vivo and have been demonstrated to be feasible and effective for clinical grade production of large numbers of T cells (Porter et al., 2006; Rapoport et al., 2005; Thompson et al., 2003). The aim of the present paper was to develop an improved protocol for adoptive T cell therapy based on the standard platform for non-selective expansion of Ag-specific T cells using Dynabeads CD3/CD28. A model system was selected based on the availability of pentamers to detect the frequencies of CMV or EBV specific CD8+ T cells before and after expansion. Following optimization of the expansion protocol for virus specific CD8+ T cells, we performed similar experiments on PBMC from a melanoma patient vaccinated with hTERT peptides, where hTERT specific CD8+ T cells could be detected prior to expansion.

In this study, we have optimized the culture conditions with the goal of obtaining high numbers of virus- and tumor specific T cells of desired phenotype after expansion with Dynabeads CD3/CD28. Removal of CD25+ cells, presumptively containing Tregs prior to expansion greatly increased the expansion of virus specific T cells (CMV and Flu). Virus- and tumor specific CD8+ T cells demonstrated an early/intermediate memory phenotype following expansion, dominated by high expression of CD62L and CD28 and low expression of CD57. By including a pre-stimulation step with peptide prior to non-specific expansion the numbers of Ag-specific T cells were dramatically increased. Together these modifications contribute to a profoundly improved protocol for expansion of Ag-specific T cells.

2. Materials and methods

2.1. Culture media, reagents, Dynabeads, antibodies and flow cytometry

T cells were cultured in CellGro® Serum-free Culture Media (CellGenix, Freiburg, Germany) supplemented with 5% heat inactivated human serum (PAA Laboratories GmbH, Austria), 10 mM N-acetylcystein (Mucomyst 200 mg/mL, AstraZeneca AS, Norway), gentamycin 0.05 mg/mL (Garamycin 40 mg/mL, Schering-Plough Europe, Belgium) and 100 U/mL recombinant human interleukin-2 (IL-2) (Proleukin®, Novartis). Recombinant human interleukin-7 (IL-7) was purchased from BD PharamingenTM. Anti-CD3/anti-CD28 coupled Dynabeads (Dynabeads® ClinExVivo™ CD3/CD28, and Dynabeads® CD3low/CD28), Dynabeads® CD4, Dynabeads® CD14, and Dynabeads® ClinExVivo™ CD25 were kindly provided by Dynal Invitrogen, Oslo, Norway. Flu vaccine was a mix of Brisbane/59/2007 strain H1N1 and Brisbane/10/2007 strain H3N2 (Solvay Biologicals BV, Netherlands). The following fluorochrome-conjugated mAbs were used for flow cytometric analyses: FITC-conjugates of anti-CD4, CD8, CD27, CD28, CD57 (Dako Cytomation, Denmark), anti-CD3, CD14 (BD Biosciences, San José, CA), anti-CCR7 and CD62L (R&D Systems, Minneapolis, MN); phycoerythrin (PE) conjugates of anti-CD4, CD25 (Dako Cytomation, Denmark); PE-Cy-5 conjugate of anti-CD8 (Dako Cytomation, Denmark); APC-conjugate of CD25, PerCP-conjugate of CD4; R-PE labeled HLA A*0201 pentamers specific for CD8+ CMVpp65 (NLVPMVATV), EBV BMLF1 (GLCTLVAML), and hTERT (ILAKFLHFLWL) were purchased from ProImmune Ltd (UK). Cells were stained with fluorochrome-labeled mAbs for 20 min at room temperature in 50 µL staining buffer (PBS + 0.1% human serum albumin (Octapharma, Stockholm, Sweden) + 0.1% Na3cit) and 5 µL of 10 mg/mL gammaglobulin (Gammagard, Baxter, UK). Pentamer staining was performed as described by the manufacturer. Briefly, cells were stained with pentamers for 15 min at room temperature prior to staining with other mAbs as described above. Samples were analyzed on a FACSscan or LSR II cytometer (Becton Dickinson, Franklin Lakes, NJ) using FlowJo software (Tree Star Inc.).

2.2. PBMC collection and cell processing

Leukapheresis was performed on patients after informed consent. The study was approved by the Norwegian Medicines Agency, the Norwegian Department of Health (Gene therapy board), the Regional Committee for Medical Research Ethics, and the Hospital Internal Review Board. It was performed in the compliance with the World Medical Association Declaration of Helsinki. Following leukapheresis, lymphocytes were enriched by elutriation (Elutra Cell Separation System, Gambro BCT). PBMC were separated by density gradient centrifugation (Lymphoprep™, Axis-Shield, Norway) and monocytes were depleted using Dynabeads CD14 as described by the manufacturer. PBMC used for expansion of virus specific T cells were collected from HLA-A2 positive healthy donors (n = 5) and stage III malignant melanoma patients (n = 4) enrolled in dendritic cell (DC) vaccine clinical trials. Expansion of tumor specific T cells was performed on PBMC from a stage IV malignant melanoma
patient, isolated from blood samples taken 4 weeks following vaccination. The patient was vaccinated with the HLA-A2 binding hTERT peptide (548–548; ILAKFLHWL) in combination with the promiscuous hTERT helper epitope (611–626; EARPALLTSRLFIPK). After vaccination and prior to expansion, the patient displayed 0.54% hTERT + CD8 + T cells, while no detectable levels of hTERT + CD8 + T cells was detected prior to vaccination.

Prior to expansion of virus specific T cells, CD25 + cells were depleted using Dynabeads CD25 following the manufacturer's instructions. Briefly, the CD4 + T cell numbers were determined by flow cytometry at the time of leukapheresis and Dynabeads CD25 were added at a CD4 + T cell-to-beads ratio of 2:1 in thawed samples (assuming 2.5–5% CD4 + CD25hi T cells, which gives 10–20 beads per target cell). Incubation was performed at 4 °C for 30 min and CD25 positive cells were captured with a magnet (MPC-6, Invitrogen Dynal, Norway).

2.3. T cell stimulation and expansion

Monocyte-depleted PBMC were stimulated with two different types of beads; Dynabeads ClinExVivo CD3/CD28 and Dynabeads CD3low/CD28. T cells (1 × 10⁶ CD3 + T cells/well) were cultured in 24-well tissue culture plates at 37 °C, 5% CO₂ at a concentration of 0.7 × 10⁶/mL. When cell numbers reached 1.7 × 10⁹/mL the cells were transferred to culture flasks (Nunclon™Surface, Nunc™, Denmark). From day 3 the cells were counted daily and the viability was evaluated by Trypan Blue (Gibco, Invitrogen, CA) staining. Fresh media was added daily to maintain a cell concentration of 0.5 × 10⁶ cells/mL. Flow cytometric analysis was performed before activation, on days 7 and 10 of the expansion. The number of Ag-specific CD8 + T cells was calculated as percentage of the total number of CD8 + cells.

In separate experiments, PBMC were stimulated with CMVpp65 (NLVPVMATV) or hTERT (ILAKFLHWL) peptides at a final concentration of 5 µg/mL (Proimmune Ltd, UK) and cells were cultured for 10–14 days in media supplemented with 20U/mL IL-2 and 5 ng/mL IL-7.

2.4. Proliferation assay

To measure proliferation of Ag-specific CD8 + T cells, CD4 + T cells were depleted using Dynabeads CD4 as described by the manufacturer. CD8 + T cells (10⁵/well) were stimulated in triplicates with peptide using irradiated autologous PBMC or LCL-B cells (0.5 × 10⁵/well) as antigen presenting cells (APCs). After 2 days, cells were pulsed with 1 µCi of ³H-thymidine over night and counted using a β-counter (Packard, Laborol, Norway). CD8 + T cells incubated with unloaded, irradiated APCs were used as negative control. To measure the proliferative response against Flu in vaccinated donors, 1 × 10⁵ PBMC or CD25 depleted PBMC were stimulated in triplicates with Flu vaccine for 6 days (final concentration 1/600) before adding 1 µCi of ³H-thymidine. Cells were harvested on day 7 and proliferation was measured using a β-counter. Stimulation index (SI) is calculated by dividing cpm of a positive sample with cpm of the negative control. SI values above 2 are considered as positive.

2.5. Titration of Dynabeads CD25 for Treg depletion in Flu vaccinated donors

Given the chronic nature of tumors and CMV infections, one might expect that CMV could provide a model for optimizing culture conditions for expansion of tumor specific T cells ex vivo. However, patients vaccinated with TAA-specific peptides might generate anti-tumor responses from precursors within the naïve pool resulting in a population of tumor specific T cells expressing CD25 following activation. The CD25 depletion step implemented for Treg removal may also deplete CD25 effector cells presumably present in PBMC after vaccination. To investigate whether Treg depletion using Dynabeads CD25 removes effector CD4 + or CD8 + T cells, healthy volunteers were vaccinated with Flu and blood samples were taken four weeks following vaccination. PBMC were stained with CD4-F and CD25-APC and the number of CD4 + CD25hi was calculated by flow cytometry prior to depletion of CD25 + cells. Depletion was performed by incubating 1 × 10⁷ PBMC in 1 mL of CellGro medium with different amounts of Dynabeads CD25 (5, 10 and 30 Dynabeads CD25/CD4 + CD25hi T cells). Cells and beads were incubated for 30 min at 2–8 °C and non-rosetted cells were removed with a magnet. CD25 depleted and non-depleted PBMC were tested in a proliferation assay as described above.

2.6. Statistical analyses

Data for Figs. 1 and 3 were analyzed by SPSS statistical software (LEAD Technologies, Inc). Intergroup comparisons were performed using Wilcoxon Signed Ranks Test (nonparametric, two-related-samples test) and significance of CD25 depletion (Fig. 5) was calculated using two tailed paired Student’s t-test. All p values were considered significant if less than 0.05.
3. Results

With the aim of developing a protocol for expansion of human tumor specific T cells, we have tested different culture conditions in order to conserve Ag-specific T cells during polyclonal expansion with Dynabeads CD3/CD28. We have explored the effect of modifying the amount of CD3 and CD28 mAbs coated onto the Dynabeads, the effect of removing CD25 positive cells prior to expansion, and the effect of including a pre-stimulation step with peptide to increase the total amount of Ag-specific T cells in the final product.

3.1. Depletion of CD25+ cells increases Ag-specific CD8+ T cell expansion

Based on previous data (Mesel-Lemoine et al., 2006) showing that Tregs inhibit the proliferative response of both CD4+ and CD8+ T cells in vitro, we wanted to investigate whether removal of CD25hi cells prior to T cell expansion could increase the final cell yield of virus specific CD8+ T cells (EBV and CMV), as well as polyclonal T cells. Non-depleted and CD25 depleted T cells from nine donors were expanded for 10 days with a bead-to-T cell ratio of 1:1 and the number of virus specific CD8+ T cells was monitored with pentamers on days 0 and 10. The fold expansion of virus specific CD8+ T cells was calculated by dividing the total number of Ag-specific CD8+ T cells in final product with the number of Ag-specific CD8+ T cells in the starting material. As shown in Fig. 1 the mean fold expansion increased from 73 to 178 (2.4 fold) after removal of CD25+ cells, (p = 0.011) and the total fold expansion of T cell increased from 230 to 315 (1.5 fold), (p = 0.008), indicating that CD25 positive Tregs were depleted following incubation with Dynabeads CD25. CD4+CD25+ Tregs (measured by Foxp3 staining) did not reappear during culture following depletion with Dynabeads CD25 and 10 days expansion (data not shown).

3.2. Ag-specific T cells were conserved after 10 days expansion with Dynabeads CD3/CD28

In order to test whether polyclonal expansion using Dynabeads conserves Ag-specific T cells, CD25 depleted T cells were incubated with Dynabeads CD3/CD28 at a bead-to-T cell ratio of 1:1 for 10 days and the number of virus specific CD8+ T cells was measured with pentamers from both groups were combined. CMV and EBV specific CD8+ T cells after 10 days (healthy donors, n = 5 and cancer patients, n = 4). Since no significant difference was observed in the fold expansion of virus specific CD8+ T cells of healthy donors and cancer patients the data from both groups were combined. CMV and EBV specific CD8+ T cells were expanded 161 fold (range 27–348, SD = 139) and 137-fold (15–324, SD = 136), respectively. A 342-fold expansion of total T cells (range 117–774, SD = 192) was observed.

Expansion curves show that in order to obtain high numbers of both polyclonal T cells and Ag-specific T cells, cells should be expanded for 9–10 days, since logarithmic growth is not apparent until day 7 after culture initiation (Fig. 2B).

3.3. The ratio of CD3/CD28 mAbs on Dynabeads influences the CD4/CD8 ratio after expansion

Kalamasz et al. have demonstrated that the fold expansion of Ag-specific CD8+ T cells can be increased by reducing the ratio of CD3/CD28 mAbs on Dynabeads (Kalamasz et al., 2004). Based on this observation we tested two types of Dynabeads with different ratio of anti-CD3/anti-CD28: Dynabeads ClinExVivo CD3/CD28 and a customized product; Dynabeads CD3low/CD28. We did not observe a preferential expansion of Ag-specific CD8+ T cell by reducing the amount of anti-CD3 on the beads (CD3low/CD28 beads, data not shown). However, the fold expansion of the CD8+ T cell population was higher when T cells were stimulated with Dynabeads ClinExVivo CD3/CD28. In contrast, when T cells were stimulated with Dynabeads CD3low/CD28, the expansion of CD4+ T cells was increased (Fig. 3), indicating that the amount of CD3 and CD28 mAbs on Dynabeads influences the outcome of CD4+ and CD8+ T cells in the final product.
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The percentages of CD4+ and CD8+ T cells were measured after 10 days or CD25 depleted) were stained with 
vivo persistence and survival. Expanded T cells (non-depleted 

3.4. Expanded CMV specific T cells comprise a mixture of cells at different stages of differentiation and harbor proliferative potential

Long-term ex vivo expansion protocols for tumor specific T cells usually generate a population of cells expressing terminally differentiation markers and show low proliferative potential in vivo (Gattinoni et al., 2005; Huang et al., 2005). However, a rapid expansion protocol performed in 12–14 days generated TILs that have features associated with in vivo persistence and anti-tumor activity (Tran et al., 2008). We wanted to test whether the 10 days expansion protocol could generate Ag-specific cells expressing markers important for in vivo persistence and survival. Expanded T cells (non-depleted or CD25 depleted) were stained with fluorochrome-labeled mAbs against defined differentiation markers, such as CD27, CD28, CD62L, CCR7, and CD57, and the percentage of CMV specific CD8+ T cells expressing the different markers was determined by flow cytometry. Prior to expansion, CMV specific CD8+ T cells demonstrated high expression of CD57 and low to intermediate expression of most other marker tested as shown in Fig. 4A. Following expansion, the percentage of Ag-specific T cells expressing CD57 was reduced, indicating preferential expansion of CD57 negative cells. The costimulatory molecule CD28, which is associated with high proliferative potential, was highly expressed following expansion probably due to enrichment for T cells expressing this molecule. The percentage of cells expressing CD62L was increased, indicating a switch from terminally differentiated T cells to a more early/intermediate memory population. Our data show that following ex vivo expansion with Dynabeads, a higher percentage of the Ag-specific CD8+ T cells express markers critical for in vivo homing and survival. Non-depleted and CD25 depleted cells showed the same differentiation phenotype following expansion.

To investigate the correlation between the phenotypic profile of expanded CMV specific T cells and the capacity to proliferate in response to antigen, we tested T cells in a restimulation assay. Expanded CMV specific T cells showed a proliferative response against CMV peptide loaded PBMC (Fig. 4B), which correlated with the early/intermediate memory phenotype shown in Fig. 4A. The mean number of CMV specific T cells in each well is given in parentheses.

3.5. CD25 depletion increases the proliferative response in Flu vaccinated donors

It has been demonstrated that depletion of Tregs from TILs prior to adoptive transfer improved the anti-tumor immunity in mice and greatly enhanced Th1 type immune response (Xu et al., 2009). However, in recently vaccinated patients CD25 expression can be upregulated on Ag-specific T cells, which increase the chance of removing vaccine induced T cells by

Fig. 3. CD4/CD8 ratio in the final product was modified when the ratio of CD3:CD28 mAbs on Dynabeads was manipulated. T cells were stimulated for 10 days with two types of Dynabeads with different ratios of anti-CD3/anti-CD28 mAbs: Dynabeads ClinExVivo CD3/CD28 and Dynabeads CD3high/CD28. The percentages of CD4+ and CD8+ T cells were measured after 10 days expansion and the CD4/CD8 ratio was calculated (n = 9). The box plot shows the CD4/CD8 ratio before and after 10 days expansion with the two types of Dynabeads. Horizontal bar in the boxes represents the median. Lower and upper whiskers are minimum and maximum values, respectively. Lower and upper limits of the boxes represent 25th and 75th quartiles, respectively. Comparison between the two types of Dynabeads was performed by two-related-samples test (Wilcoxon test).

Fig. 4. Expanded CMV specific CD8+ T cells display a mixture of cells at different stages of differentiation and the cells show proliferative potential. Non-depleted and CD25 depleted T cells from three donors containing detectable numbers of CMV+CD8+ T cells were expanded for 10 days with Dynabeads CD3/CD28 as described in "Materials and methods". Following expansion the phenotypic profile of CMV+CD8+ T cells was determined by flow using mAbs against CD27, CD28, CCR7, CD62L, and CD57. A: Data represent mean percentage of CMV+CD8+ T cells expressing the different markers (n = 3). B: Expanded T cells depleted of CD4+ T cells were stimulated with CMV peptide loaded PBMC and the proliferative response was measured on day 3. T cells incubated with unloaded PBMC were used as a negative control. The parentheses on the bar plots show the mean number of CMV+CD8+ T cells per well (n = 3). Error bars indicate SD.
CD25 depletion. We wanted to explore the effect of removing CD25+ cells in a vaccination setting before implementing a step for depletion of CD25+ cells prior to T cell expansion in a clinical protocol. Due to limited availability of patient samples expressing tumor specific CD8+ T cells that can be monitored by pentamers, we vaccinated healthy donors with Flu and compared the proliferative capacity of Ag-specific T cells before and after CD25 depletion in samples taken four weeks following vaccination. Removal of CD25+ cells increased the proliferative response against Flu, indicating efficient depletion of Tregs while sparing Flu-specific T cells (Fig. 5). These data correlated with the increased expansion observed for CMV and EBV specific T cells (Fig. 1).

3.6. Pre-stimulation with peptide prior to Dynabeads CD3/CD28 expansion increases the final yield of hTERT specific CD8+ T cells

One obstacle for ex vivo expansion of tumor specific T cells is the low precursor frequency of Ag-specific T cells in PBMC, which usually leads to low numbers of Ag-specific T cells in the final product. As documented by Dang et al. (2007) a pre-stimulation step with peptide prior to Dynabeads expansion increased the total number of Ag-specific T cells in the final product. Based on these data we compared the total yield of Ag-specific CD8+ T cells in PBMC pre-stimulated with peptide prior to Dynabeads expansion. PBMC from the malignant melanoma patient vaccinated with hTERT peptides were incubated with hTERT (ILA) peptide for 14 days in the presence of IL-2 and IL-7 prior to expansion with Dynabeads. As shown in Fig. 6 the fold expansion of hTERT specific CD8+ T cells was significantly higher when a pre-stimulation step with peptide was included (4714 fold) compared to directly expanded cells (44 fold).

3.7. Expanded hTERT specific T cells comprise a mixture of cells at different stages of differentiation

CMV specific CD8+ T cells demonstrated an early/intermediate memory phenotype following expansion with Dynabeads. To investigate whether this was a phenomenon related only to ex vivo expansion of virus specific T cells or could be applied to tumor specific T cells, we monitored the phenotype of hTERT specific CD8+ T cells before and after expansion. The data presented in Fig. 7A demonstrated that tumor specific T cells express high levels of CD27 and CD57 and low levels of most other marker tested prior to expansion. Following pre-stimulation with hTERT peptide (ILA) the Ag-specific CD8+ T cells upregulated the expression of CD28 and CD62L and the expression of CD57 was strongly reduced (Fig. 7A). Further expansion with Dynabeads generated hTERT specific CD8+ T cells with a similar phenotype (Fig. 7B). The same phenotypic profile was also detected on hTERT+CD8+ T cells directly expanded without a pre-stimulation step. Taken together, the hTERT specific CD8+ T cells expressed an early/intermediate memory phenotype following 10 days ex vivo expansion with Dynabeads.

3.8. Ex vivo expanded hTERT specific T cells are proliferative

To analyze the proliferative capacity of hTERT specific CD8+ T cells we tested expanded hTERT specific CD8+ T cells against LCL-B cells loaded with hTERT peptide. T cells pre-stimulated with peptide prior to expansion demonstrated a higher proliferative response (SI=8.7) than T cell expanded without a pre-stimulation step (SI=2.8), probably explained by the higher number of hTERT specific T cells in the pre-stimulated sample (Fig. 7C).

4. Discussion

Protocols for ex vivo expansion of human T cells have been developed for clinical use and adoptive transfer of expanded T cells has shown promising results in a number of cancer patients (Thompson et al., 2003; Porter et al., 2006). However, several protocols for expansion of Ag-specific T cells require long-term ex vivo expansion, generating terminally differentiated effector cells which do not persist.
The protocol presented in this paper has several advantages; first, expansion is performed in ten days or 20–24 days if a pre-stimulation step is included, which is shorter than the 45–60 days expansion protocols for CTLs (Topalian et al., 1987).

Secondly, expanded Ag-specific T cells expressed an early/intermediate memory phenotype as demonstrated by high expression of CD62L and CD28 and low expression of CD57. Interestingly, this was also the case when the Ag-specific T cell population in the starting material was dominated by terminally differentiated cells.

A critical step in adoptive T cell therapy is trafficking of adoptively transferred tumor specific T cells to lymph nodes. This is facilitated by up-regulation of the expression of CCR7 and CD62L, both known to be important for entry into secondary lymphoid tissue. Interestingly, we found a high percentage of cells expressing CD62L and few cells expressing CCR7, consistent with recently published data (Decrion et al., 2007) demonstrating the existence of an intermediate population of CMV specific CD8+ T effector memory cells. Furthermore, in a recent paper Berger et al. (2007) show that in macaques, antigen-specific TCM cells, but not TEM cells, persisted long-term in vivo, reactivated phenotypic and functional properties of memory T cells, and occupied memory niches. Although the T cell clones had differentiated to effector cells during 49 days culture, T cell clones generated from the central memory pool re-expressed CD62L, CCR7, CD28 and CD27 after transfer in vivo. In a recent paper, Perret and Ronchese (2008) demonstrated that the adoptive transfer of a mixed population of T cells had the benefit of contributing the direct killing effect mediated by effector cells and TCM and the homing feature of TEM which can traffic to LN where they can proliferate after interaction with incoming DC from the tumor site. The protocol described here for expansion of Ag-specific T cells generated a mixture of cells at different levels of differentiation which might be optimal for adoptive transfer. It has been demonstrated that T cells which do not reach a state of terminal differentiation during ex vivo culture, are able to survive and convert to a memory phenotype in vivo and persist long term as TEM (Powell, Jr. et al., 2005).

Another important factor for clinical outcome is the total number of Ag-specific T cells present in the final T cell product. This number depends on the clonal expansion of Ag-specific T cells in vivo, prior to T cell harvesting, and the culture conditions. We tried to improve Ag-specific expansion by two different strategies; firstly we removed CD25+ cells prior to T cell expansion and secondly we introduced an antigen pre-stimulation step ex vivo prior to expansion. We found that depletion of CD25+ cells prior to T cell expansion increased the number of virus specific T cells generated during culture. Increased numbers of Treg are found in peripheral blood and tumor draining lymph nodes in late stage cancer patients, correlating with decreased anti-tumor response and reduced survival. Additionally, the inclusion of a CD25 depletion step for removal of Tregs prior to ex vivo expansion might improve the outcome of adoptive T cell therapy since bulk transfer of T cells containing Tregs has been demonstrated to inhibit the anti-tumor immune response (Xu et al., 2009). Based on the observation that CD25 depletion led to increased expansion of CMV and EBV following adoptive transfer (Huang et al., 2005; Rosenberg et al., 2003). In an attempt to develop a shorter expansion protocol generating high numbers of tumor specific T cells with the ability to survive and persist in vivo we have optimized a protocol for polyclonal expansion of T cells using Dynabeads CD3/CD28.
specific CD8+ T cells probably due to depletion of Tregs, we further tested whether similar results could be obtained in recently vaccinated donors. In samples taken four weeks after vaccination we detected an increased Flu-specific T cell proliferation, indicating that removal of CD25+ Tregs can be used prior to expansion in vaccinated patients. However, in order to prevent removal of vaccine induced effector T cells by CD25 depletion, patient samples should be tested at different time points following vaccination prior to depletion and expansion.

Different kinds of regulatory cells may negatively influence the immune response in cancer patients and CD4+CD25+ Tregs may not be the only target to manipulate in future immunotherapy trials. One candidate is myeloid-derived suppressor cells (MDSC) which have been demonstrated to inhibit T cell function directly (Kusmartsev and Gabrilovich, 2006) as well as via induction of Tregs (Hoechst et al., 2008). These cells are however not present in our in vitro culture systems, since they have been removed by elutriation prior to culture. In a recent paper, Ko et al. (2009) show that sunitinib, a receptor tyrosine kinase inhibitor significantly reduces the number of MDSC in renal cell carcinoma patients. This drug may thus prove particularly useful in combination with cancer vaccines. CD8+ regulatory T cells have also been identified in cancer patients. In a recent paper, Andersen et al. (2009) describe a naturally occurring HLA-A2-restricted CD8+ T cell specific for the anti-inflammatory molecule heme oxygenase-1. These cells had a superior suppressive effect on both CD4+ and CD8+ T cells in vitro and the potential for manipulating these types of regulatory cells has so far not been investigated in the context of ex vivo T cell expansion.

We further found that peptide stimulation prior to expansion with Dynabeads CD3/CD28 greatly facilitated generation of high numbers of Ag-specific T cells. Even in donors with low frequency of Ag-specific CD8+ T cells in the starting material significant numbers of tumor specific T cells could be generated using this approach. Our results are in line with a recent publication showing that vaccination combined with short-term peptide-specific ex vivo stimulation followed by CD3/CD28 Dynabeads expansion greatly increased the number of Ag-specific T cells (Dang et al., 2007). Furthermore, recent experiments in mice (Ghosh et al., 2008) demonstrated that ex vivo stimulation of T cells using tumor lysate-pulsed DCs followed by expansion using Dynabeads CD3/CD28 generated high numbers of leukemia-specific CTLs that cured 80% of mice bearing leukemia. An inclusion of a pre-stimulation step with DC loaded with tumor mRNA or tumor lysate could thus easily be included prior to ex vivo expansion of T cells with Dynabeads in patients taking part in clinical protocols involving DC vaccination. This may also be a more generally applicable modification of this method.

Taken together these results indicate that a combination of Treg depletion and ex vivo antigen stimulation may constitute important contributions to more efficient T cell expansion protocols for adoptive transfer of T cells.

In contrast to the previous study reporting increased proliferation of Ag-specific CD8+ T cells by reducing the amount of anti-CD3 on the beads (Kalamasz et al., 2004), our data showed no differences in the fold expansion of Ag-specific CD8+ T cells. However, we observed an increased CD4/CD8 ratio when the amount of anti-CD3 on Dynabeads was reduced, indicating that manipulating the ratio of anti-CD3 and anti-CD28 on beads may influence expansion of CD4+ versus CD8+ T cells. We have optimized our protocol based on monitoring the expansion of Ag-specific CD8+ T cells in HLA-A2 positive donors due to limited availability of HLA class II ulitmers/tetramers. The expansion of CD4+ T cells, however may be important based on preclinical and clinical studies showing that CD4+ T cells play a critical role in generating anti-tumor effects (Li et al., 2002). Recently, it has been reported that a melanoma patient with a NY-ESO-1 specific CD4+ T cell clone generated durable clinical remission and led to immune responses to other melanoma specific antigens displayed by the tumor (Hunder et al., 2008). Therefore, the transfer of expanded tumor specific CD8+ T cells generated during vaccination and expanded ex vivo could sustain the tumor specific CD8+ T cells’ persistence in vivo and improve the clinical outcome.

In summary our data demonstrate that large numbers of Ag-specific T cells expressing markers important for in vivo survival can be generated for adoptive T cell therapy by optimizing the ex vivo culture conditions. The use of a cancer vaccine as an initial step to prime tumor specific T cells combined with ex vivo priming and expansion of T cells represents an attractive immunotherapeutic approach that may be more effective clinically than previously used therapies. Based on our results we have developed a cGMP compatible protocol for large scale production of T cells using Dynabeads CD3/CD28 and Wave Bioreactor (Almåsbak et al., paper in preparation) and a clinical protocol has been approved combining mRNA loaded DC vaccination and ex vivo expanded tumor specific T cells in patients with advanced stage melanoma. We believe that the described method for T cell expansion may be of interest not only in the context of clinical trials of T cell therapy, but also as a powerful method to expand Ag-specific T cells for in vitro studies.

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