Successful generation of primary virus-specific and anti-tumor T-cell responses from the naïve donor T-cell repertoire is determined by the balance between antigen-specific precursor T cells and regulatory T cells

Inge Jedema,[1] Marian van de Meent,[1] Jeanette Pots,[1] Michel G.D. Kester,[1] Martha T. van der Beek,[2] and J.H. Frederik Falkenburg[1]

[1]Department of Hematology, Laboratory of Experimental Hematology, Leiden University Medical Center, Leiden; [2]Department of Medical Microbiology, Leiden University Medical Canter, Leiden, The Netherlands

ABSTRACT

Background
One of the major challenges in allogeneic stem cell transplantation is to find a balance between the harmful induction of graft-*versus*-host disease and the beneficial graft-*versus*-leukemia and pathogen-specific immune responses. Adoptive transfer of *in-vitro* generated donor T cells with specific anti-leukemic or pathogen-specific activity may be effective. However, in many cases this requires the *in-vitro* priming and expansion of antigen-specific precursor T cells from the naïve donor T-cell repertoire.

Design and Methods
Antigen-specific CD8 T cells were generated by co-culture of CD45RO-depleted, regulatory T cell-depleted donor peripheral blood mononuclear cells with autologous peptide-loaded dendritic cells, followed by two re-stimulations with peptide-loaded autologous monocytes. Responding T cells were isolated based on CD137 expression and further purified using peptide/major histocompatibility complex tetramers.

Results
Using this method we were able to reproducibly generate functionally high avidity T cells directed against multiple viral antigens and minor histocompatibility antigens from the naïve T-cell repertoire of seronegative, minor histocompatibility antigen-negative donors. Furthermore, we demonstrated that reduction of the regulatory T-cell frequency by depletion of $CD45RO^+$ responder cells resulted in improved priming and expansion of antigen-specific precursor T cells.

Conclusions
In conclusion, we present a robust method for the *in-vitro* induction and isolation of antigen-specific T cells from the naïve repertoire. We demonstrate that the likelihood of successful generation of primary immune responses is determined by a delicate balance between the numbers of antigen-specific precursor T cells and the numbers and activation state of regulatory T cells locally at the site of priming of the immune response.

Key words: primary immune responses, regulatory T cells, CMV, minor histocompatibility antigens, immunotherapy.

Citation: Jedema I, van de Meent M, Pots J, Kester MGD, van der Beek MT, and Falkenburg JHF. Successful generation of primary virus-specific and anti-tumor T cell responses from the naïve donor T-cell repertoire is determined by the balance between antigen-specific precursor T cells and regulatory T cells. Haematologica 2011;96(8):1204-1212. doi:10.3324/haematol.2010.039099

Funding: this work was supported by a grant from the 6th Framework Program of the European Union (AlloStem, EU grant 503319)

Manuscript received on December 15, 2010. Revised version arrived on April 8, 2011. Manuscript accepted on April 26, 2011.

Correspondence:
Inge Jedema, PhD
Leiden University Medical Center, Dept. of Hematology, C2R-140, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
Phone: international +31-71-526 2271
Fax: international +31-71-526 6755
E-mail: i.jedema@lumc.nl

The online version of this article has a Supplementary Appendix.

Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is commonly used in the treatment of patients with hematologic malignancies.[1] Graft-*versus*-host disease is the main complication after transplantation, and T-cell depletion of the stem cell graft results in a significant reduction of the incidence of acute graft-*versus*-host disease. However, since donor T cells are also the main effectors of the beneficial graft-*versus*-leukemia effect after allogeneic SCT, additional strategies are necessary to support this effect.[2,3] Moreover, pathogen-specific T cells are necessary for adequate protection of patients against viral and fungal infections.[4] Indeed, infections by human cytomegalovirus (CMV) are a major cause of morbidity and mortality after T-cell-depleted allogeneic SCT.[5,6] Ultimately, immunological reconstitution of T cells derived from the hematopoietic system of donor origin will be achieved, but it may take more than a year for a full T-cell repertoire to be formed. In addition, donor T cells maturing from the stem cell graft are educated in the patient's environment, leading to the induction of patient-specific tolerance, thereby limiting these cells' potential anti-leukemic activity. This warrants the exploration of strategies using donor-derived T cells to supply an adequate graft-*versus*-leukemia effect and anti-viral immunity in the first year after allogeneic SCT.

Application of unmodified donor lymphocyte infusion after allogeneic SCT has been demonstrated to result in graft-*versus*-leukemia responses and transfer of protective immunity against CMV, Epstein-Barr virus (EBV) and other pathogens but may also induce severe graft-*versus*-host disease, even at low T-cell doses[7-9] *In-vitro* induction and selection of donor T cells with specific anti-leukemic or pathogen-specific activity may be an attractive strategy to increase both the specificity and effectiveness of adoptive transfer of donor T cells, allowing safe application in immunocompromised patients after allogeneic SCT. Different strategies have been developed to purify specific T-cell populations, either based on their specific proliferation, cytotoxicity, and/or cytokine production after *in-vitro* stimulation with the relevant antigen(s) or by direct isolation based on their T-cell receptor (TCR) specificity using specific peptide/MHC multimers.[10-19] Indeed, adoptive transfer to patients within the first months after transplant of virus-specific memory T cells isolated from seropositive donors has been reported to be safe and effective.[10,13,16,20]

However, if no specific immunological memory response against the target antigens is present in the donors, the induction of a primary immune response is required, for instance to isolate CMV-specific T cells from CMV-seronegative donors. In general, donor T cells targeting antigens that are expressed on the (malignant) hematopoietic cells of the patient, e.g. minor histocompatibility antigens (mHag), also have to be isolated from the naïve T-cell repertoire. Because of the very low frequencies of antigen-specific precursor T cells (Tprec), enrichment of antigen-specific T cells from primary immune responses to be used for adoptive transfer requires alternative strategies. Although successful *in-vitro* induction and isolation of antigen-specific T cells from primary immune responses have been reported,[21-25] the large number of variables determining the likelihood of successful induction of primary immune responses has hampered the reproducible production of enriched populations of antigen-specific T cells for adoptive transfer. Several factors may responsible for this variability, including the very low frequencies of antigen-specific Tprec in the naïve donor T-cell repertoire, disturbance or overgrowth caused by off-target bystander activation of neighboring cells, an inappropriate cytokine milieu, inappropriate antigen presentation, or the active inhibition of the priming of antigen-specific Tprec by regulatory T cells (Treg).

In this paper we describe the *in-vitro* induction of antigen-specific primary immune responses and present a strategy resulting in the reproducible induction and expansion of antigen-specific T cells from the naïve donor T-cell compartment.

Design and Methods

Donors

Peripheral blood from healthy donors was harvested after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density separation and cryopreserved for further use. Donors positively typed for HLA-A*0101, A*0201, A*2402, B*0702, and/or B*0801 by high resolution genomic DNA typing and with a negative serostatus for CMV were selected.

Selection of cell populations

The selection of cell populations is described in the *Online Supplementary Appendix*.

Analysis of T-cell activation kinetics

To analyze the kinetics of activation of naïve T cells, memory T cells and Treg, the cells were exposed to either autologous or HLA-mismatched allogeneic monocyte-derived dendritic cells at a 1:1 ratio, or were polyclonally activated using CD3/CD28 T-cell activation beads (Dynabeads, Invitrogen, Life Technologies, Breda, The Netherlands) at a 10:1 cell to bead ratio in Iscove's modified Dulbecco's medium (IMDM) containing 10% human serum. T-cell activation was measured by flow cytometric analysis of the expression of T-cell activation markers, as described below, on days 1, 2 and 3 after stimulation.

Peptides and tetramers

Information on the specific peptides and the generation of tetramers is provided in the *Online Supplementary Appendix*.

Activation and enrichment of antigen-specific T cells

Autologous monocyte-derived antigen-presenting cells (APC) were exogenously loaded for 2 h at 37°C with 1 μM of each relevant peptide, followed by three washing steps. CD14- or CD14/CD45RO-depleted responder cells were seeded at a concentration of 1×10^6 cells/mL and co-cultured for 10 days with irradiated (25 Gy) peptide-loaded APC at a 10:1 responder to stimulator (R:S) ratio in IMDM containing 10% prescreened human serum and supplemented with 5 ng/mL human interleukin (IL)-7 (Biosource/Invitrogen, Breda, The Netherlands). In a selected number of experiments 5 ng/mL IL-15 and/or IL-21 (Biosource) were also added. On day 10 of the immune response the antigen-specific T cells were specifically re-stimulated with irradiated (25 Gy) peptide-loaded (1 μM) autologous monocytes at a 10:1 R:S ratio. On day 20 of the immune response a second re-stimulation step with irradiated (25 Gy) peptide-loaded (1 μM) autologous monocytes was performed (R:S ratio, 10:1), followed by isolation of the responding cells based on their induced surface expression of CD137. Cells were labeled with allophycocyanin-conjugated CD137 antibodies (clone 4B4-1, BD biosciences, Breda, The Netherlands) for 30 min at 4°C, washed and isolated by magnetic bead separation using anti-allophycocyanin beads according to the

manufacturer's instructions (Miltenyi Biotec). Post-isolation expansion was induced using an allogeneic feeder mixture consisting of culture medium, 5 x irradiated allogeneic feeder cells, 0.5 x irradiated allogeneic EBV-induced lymphoblastoid cell lines (EBV-LCL), 100 IU/mL IL-2, and 800 ng/mL phytohemagglutinin (PHA, Murex Biotech Limited, Dartfort, UK), or in a selected number of experiments using a feeder mixture consisting of culture medium, 5 x autologous feeder cells (CD14-negative PBMC fraction), 5 ng/mL IL-7, 5 ng/mL IL-15, and 0.2 x CD3/CD28 Dynabeads ClinEx (Invitrogen, Life Technologies). The purity of the isolated cell populations was determined by flow cytometric analysis of the frequencies of T cells stained with the relevant tetramers on day 7-10 after re-stimulation. For further enrichment steps, the antigen-specific cells were stained with phycoerythin (PE)- or allophycocyanin-labeled tetramers for 30 min at 4°C, followed by magnetic bead isolation using fluorochrome-specific magnetic beads (Miltenyi Biotec) and, 10-14 days post-isolation expansion, using an allogeneic feeder mixture.

Analysis of cytotoxicity and cytokine production

The cytotoxic capacity of the isolated antigen-specific T cells was determined in standard 6 h ^{51}Cr release assays.[26] As target cells, autologous monocytes or EBV-LCL exogenously loaded with different concentrations (1 pM to 1 μM) of the relevant peptides were used. To test the functional activity of the T cells against target cells presenting endogenously processed antigen, we used EBV-LCL that were incubated overnight at 37°C with protein-spanning overlapping peptide pools (CMV/pp65 and CMV/IE1, 1 μM) or recombinant CMV/pp65 protein (Miltenyi Biotec), or EBV-LCL transduced with a retroviral vector containing a CMV/pp65 expression construct[27] as target cells. Target cells were incubated with effector cells at an E:T ratio of 5:1. In some experiments, CMV-infected human fibroblasts were used as target cells. For this purpose, MRC-5 human fetal lung fibroblasts (American Type Culture Collection; ATCC; Wessel, Germany) were infected *in vitro* with the AD 169 human herpes virus 5/CMV strain (ATCC).

For analysis of interferon-γ production, 5,000 T cells were co-cultured with 30,000 stimulator cells. After 24 h, supernatants were harvested and the concentration of interferon-γ was measured by an enzyme-linked immunsorbent assay (Sanquin Reagents, Amsterdam, The Netherlands).

Flow cytometry analysis and proliferation/inhibition assays

Information on the methods used for flow cytometry analysis and the antibodies employed is provided in the *Online Supplementary Appendix*. Details of the assays used for the analysis of T-cell proliferation and Treg inhibition are also given in the *Online Supplementary Appendix*.

Statistical analysis

The statistical evaluation of the data was performed using the paired Student's t test.

Results

In-vitro *priming of primary immune responses by peripheral blood mononuclear cells in the presence of various cytokines*

To induce primary immune responses *in vitro* against CMV and/or mHag, PBMC from CMV-seronegative healthy donors were exposed to autologous monocyte-derived dendritic cells, exogenously loaded with mixtures of relevant 9-11-mer peptides (2-8 per response) derived from the CMV proteins CMV/pp50, CMV/pp65 and immediate early antigen 1 (CMV/IE1), and the mHag LB-ADIR-1F, LB-ECGF-1H, HA-1 and ACC1y binding to HLA-A*0101, A*0201, A*2402, B*0702 or B*0801, depending on the HLA typing of the responder cells, followed by specific re-stimulation steps. In pilot studies, we attempted to induce CMV-specific and/or mHag-specific CD8$^+$ T cells from ten CMV-seronegative, mHag-negative donors using this protocol in the presence of IL-7, IL-15 and IL-2. This did not lead to the induction of detectable populations of CD8$^+$ T cells recognizing the targeted antigens as measured by tetramers. In contrast, we observed off-target expansion of CD8$^+$ T cells, CD4$^+$ T cells, NK cells and/or γδ T cells. To determine whether the specific cytokine milieu was responsible for this off-target expansion, we tested various combinations of cytokines in the stimulation protocol in the presence or absence of exogenous antigen. Since IL-7 has been described to be responsible for the *in-vivo* maintenance and expansion of the naïve T-cell pool,[28] IL-7 was used in all cultures. Addition of IL-2 did not improve the generation of CMV-specific T cells in our system and also did not influence the composition of the cultures (*data not shown*). Both in the absence and presence of specific peptides loaded on the APC, the addition of IL-21 resulted in significant off-target expansion of γδ T cells (10.1-fold increase, n=6), and the addition of IL-15 resulted in undesired expansion of both NK cells and γδ T cells (22.3- and 4.4-fold increase, respectively, n=6). Both cytokines also induced off-target proliferation of normal αβTCR$^+$ T cells (1.3- and 2.1-fold increase for IL-21 and IL-15, respectively, n=6), resulting in overall increased numbers of CD4 and CD8$^+$ T cells. Therefore, in order to minimize off-target expansion and to avoid the risk of favoring IL-2-mediated activation of Treg, IL-7 was the only exogenous cytokine added to the cultures.

CD45RO depletion resulted in combined depletion of memory and regulatory T cells allowing in-vitro generation of primary immune responses

Since primary immune responses are derived from the naïve T-cell repertoire, depletion of CD45RO$^+$ memory T cells may limit off-target bystander proliferation, and will result in at least a 2-fold increase in the frequency of naïve Tprec. Moreover, since the majority of naturally occurring CD4$^+$/FoxP3$^+$ Treg are CD45RO$^+$,[29-32] depletion of CD45RO$^+$ cells from the responder PBMC population may also result in depletion of most Treg. We investigated the frequencies of CD4$^+$/CD25$^+$/FoxP3$^+$ Treg and CD45RA$^+$/CD27$^+$/CD62L$^+$ naïve T cells before and after CD45RO depletion. CD45RO depletion resulted in a decrease of Treg from 0.2-4.5% to only 0-0.1% (n=20). Although CD45RA$^+$/FoxP3$^+$ Treg with inhibitory potential have been described,[33,34] their numerical contribution to the total number of circulating Treg is low, resulting in almost complete removal of Treg from the responder cell population by CD45RO depletion. The frequency of naïve T cells increased after CD45RO depletion, such that these cells comprised 85-99% of the responder T-cell population, depending on the frequency of the contaminating CD45RA$^+$/CD27$^-$ late stage effector T cells. Using CD45RO depleted donor PBMC as responder cells and monocyte-derived dendritic cells loaded with multiple CMV- and mHag-specific peptides as stimulator cells, we were able to successfully activate and expand antigen-specific T cells as demonstrated by tetramer staining. Figure 1 shows tetramer

stains of representative immune responses for three individual donors. As shown in the upper dot plot in Figure 1A, no tetramer-positive cells could be identified in the naïve donor repertoire prior to the induction of the immune response. This observation was made for all responder cell populations within this cohort. In most responses, the number of tetramer-positive cells was still below the detection limit 10 and 20 days after initial priming. On day 20 of the immune response, a second re-stimulation with peptide-loaded autologous monocytes was performed and activated T cells were isolated based on their expression of the activation marker CD137 after 24 h, resulting in enrichment of virus-specific T cells (Figure 1A) and mHag-specific T cells (Figure 1B and C) with various specificities. Figure 1 shows representative examples for different specificities enriched within different immune responses. These tetramer-positive T-cell populations could be further purified using peptide/MHC multimers (*data not shown*).

Influence of CD45RO-positive regulatory T cells on the generation of primary immune responses

To investigate whether not only depletion of memory T cells but also depletion of Treg by CD45RO depletion of the responder T-cell population was relevant for the improvement of the procedure, we restored the initial frequencies of Treg prior to CD45RO depletion by adding back MACS-purified $CD4^+/CD25^{high}/CD45RO^+$ Treg to the CD14/CD45RO-depleted naïve responder population prior to the induction of the immune response. The percentages of $FoxP3^+$ cells within these isolated cell populations ranged from 40-76%. As illustrated by a representative example in Figure 2, Treg add-back strongly inhibited the induction of the immune response, demonstrated by the decreased frequencies of tetramer-positive cells (1.69%±1.82 without Treg add-back *versus* 0.154%±0.25 with Treg add-back, $P<0.05$, n=10). These differences were similarly reflected in the absolute numbers of tetramer-positive cells that were generated (4.5-fold decrease, $P<0.05$, n=10). In conclusion,

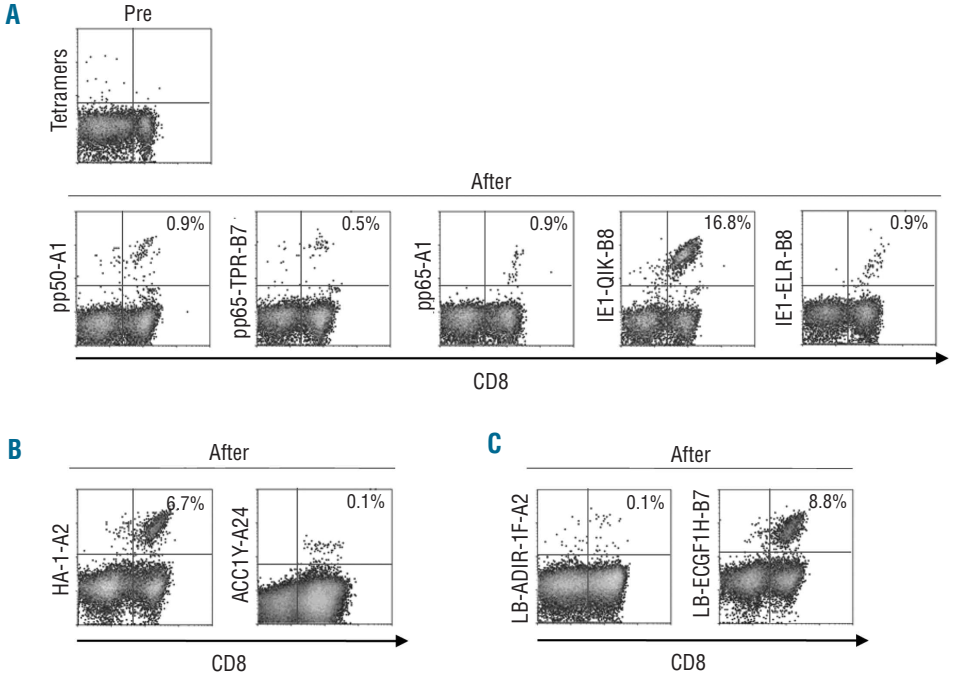

Figure 1. ***In-vitro*** **generation of virus-specific and mHag-specific T cells from a primary immune response PBMC from CMV-seronegative, mHag-negative healthy donors were depleted of $CD45RO^+$ cells by magnetic bead separation prior to co-culture with autologous monocyte-derived dendritic cells exogenously loaded with a mixture (2-8 per response) of CMV-derived and mHag-derived 9-11 mer peptides binding in HLA-A*0101, A*0201, A*2402, B*0702 or B*0801. Representative examples of tetramer stains after CD137 enrichment of responding cells on day 21 of the immune response are shown. The upper dot plot (pre) shows the absence of tetramer-positive cells prior to the induction of the immune response. The lower panels of dot plots (after) show the presence of CMV-specific T cells with different specificities in donor 1 (A) and mHag-specific T cells in donors 2 (B) and 3 (C) after *in-vitro* activation and enrichment.**

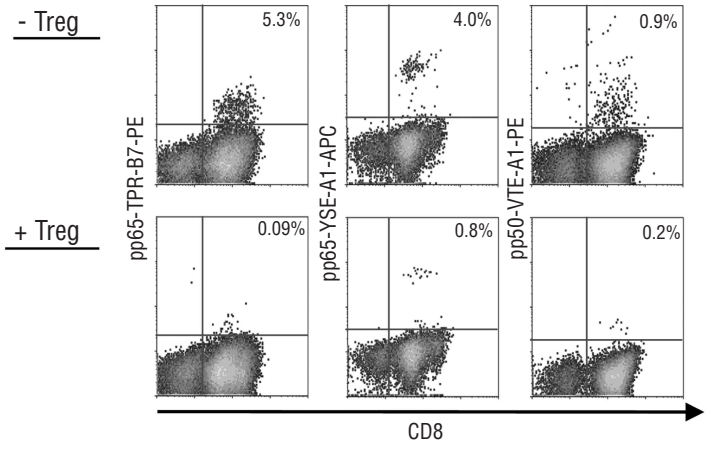

Figure 2. **Dampening of the *in-vitro* induction of antigen-specific primary immune responses by $CD45RO^+$ regulatory T cells. Primary immune responses against multiple HLA-A*0101, A*0201, B*0702 or B*0801-binding CMV peptides were induced using CD45RO-negative naïve T cells from CMV-seronegative donors as responder cells and peptide-loaded autologous dendritic cells as stimulator cells (n=4). The effect of restoring the frequency of Treg by adding back purified $CD4^+/CD25^{high}/CD45RO^+$ Treg at the onset of the immune response was analyzed. The presence of CMV-specific tetramer-positive T cells on day 30 of the immune response after *in-vitro* induction, expansion and CD137 enrichment in the absence (upper panel) or presence (lower panel) of Treg are shown for one immune response as a representative example.**

not only enrichment of naïve Tprec, but also the depletion of CD45RO$^+$ Treg contributed to the increased robustness of the procedure.

Since the frequencies of Treg in unseparated PBMC are approximately 100- to 1000-fold higher than the frequencies of Tprec specific for antigens targeted in a primary immune response, this may shift the balance towards negative regulation, whereas after CD45RO depletion the balance will change in favor of priming of the Tprec, despite the remaining minimal frequencies of CD45RO-negative naïve Treg. We hypothesized that if both the Treg and the antigen-specific Tprec were activated by the mature dendritic cells, this would explain the delicate balance between induction and regulation. As shown in *Online Supplementary Figure S1*, naturally occurring CD4$^+$/CD25^{high}/FoxP3$^+$ Treg in primary donor PBMC were not activated (panel A), but 24 h after exposure to autologous APC up-regulation of the activation markers GITR and CD137 and down-regulation of the naïve T-cell marker CD62L were observed (panel B). In addition, the activation of Treg was accompanied by a 3-fold increase in intensity of FoxP3 expression (average MFI 192±12 and 562±25, respectively, n=3). These results indicate that not only the relative numbers of Treg and Tprec, but also the kinetics of their activation may determine the balance between inhibition by Treg and priming of Tprec. Since the frequencies of antigen-specific Tprec are too low to analyze the activation kinetics after antigen-specific stimulation, we used a polyclonal stimulation via the TCR by CD3/CD28 stimulation beads and analyzed the kinetics of activation of conventional T cells and Treg. As shown by a representative example in Figure 3, 1 day after stimulation with CD3/CD28 beads, uniform up-regulation of the activation markers CD69, GITR and CD137 and down-regulation of CD62L was seen in the FoxP3$^+$ Treg population, whereas even after this strong activation signal only part of the conventional T cells was activated (Figure 3B). Figure 3C illustrates the rapid and uniform activation of Treg already within the first day after stimulation, whereas both CD45RO$^-$ naïve T cells and CD45RO$^+$ memory T cells showed more delayed and partial activation within the first days after polyclonal activation via CD3/CD28 stimulation (n=6, P<0.01).

Next, we investigated whether the activation of Treg induced by exposure to autologous APC or polyclonal activation via their TCR increased their inhibitory potential. As shown in *Online Supplementary Figure S2*, freshly isolated Treg inhibited CD3/28-induced proliferation of naïve Tprec only when they had a numerical advantage (Treg frequencies >50%) (white circles). Activation by exposure of the Treg to autologous APC increased their inhibitory potential, reflected by inhibition of Tprec proliferation at lower Treg frequencies (gray circles, P=0.03). More vigorous TCR-mediated activation of Treg mimicked by CD3/CD28 cross-linking further increased their inhibitory potential

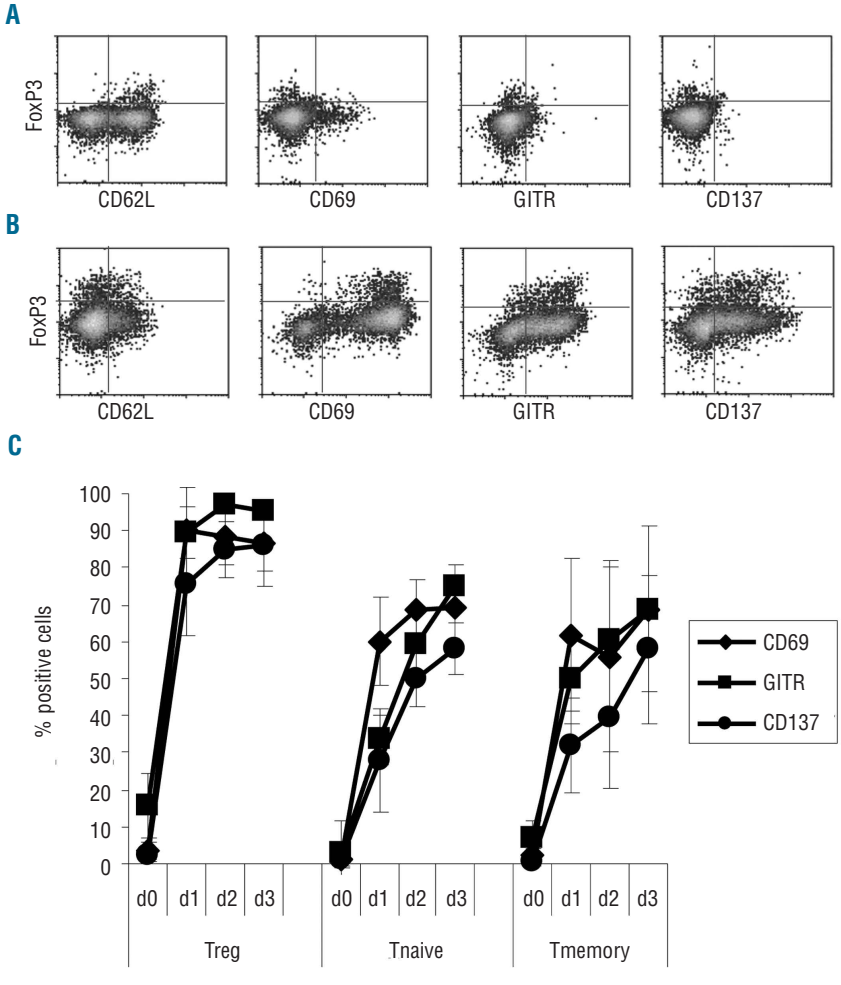

Figure 3. Differential activation kinetics of regulatory and conventional T cells. The activation state of conventional T cells and FoxP3$^+$ Treg from a healthy donor under steady state conditions (A) and 24 h after polyclonal stimulation with CD3/28 beads (B) was determined by flow cytometric analysis of co-expression of CD62L, CD69, GITR and CD137. Treg were identified by FoxP3 staining. Panel C shows the kinetics of expression of these markers on days 1, 2, and 3 after CD3/28 stimulation specifically on Treg, CD45RA$^+$ naïve T cells, and CD45RO$^+$ memory T cells (n=6).

(black circles, $P<0.01$). In conclusion, depletion of Treg prevents the local inhibition of activation of Tprec locally at the site of the APC, increasing the capacity of the dendritic cells to induce a primary immune response.

Isolation of high avidity cytomegalovirus-specific and minor histocompatibility antigen-specific T cells from the naïve T-cell compartment of cytomegalovirsu-seronegative, minor histocompatibility antigen-negative donors

The reproducibility of our method to induce primary immune responses against different CMV-derived epitopes was tested in a cohort of 36 CMV-seronegative healthy donors. CMV-specific T cells could be detected after CD137 enrichment in only 55% of the immune responses started with less than 25×10^6 CD45RO depleted donor PBMC (n=14). When the starting population consisted of more than 25×10^6 T cells (n=22), 96% of the attempts were successful in generating at least one CMV specificity, illustrating that the frequencies of Tprec specific for the targeted CMV antigens within the naïve donor T cell repertoire were low.

The results from these 22 inductions starting with more than 25×10^6 CD45RO-depleted donor PBMC are summarized in Figure 4A. The listed individual epitopes could be targeted in 13-22 separate immune responses, depending on the HLA typing of the donors, and the percentages of successful inductions per specificity ranged from 13% (2/15) to 73% (16/22). Induction of an immune response was considered successful when a population of tetramer-positive $CD8^+$ T cells consisting of more than 0.5% of the total T-cell population could be detected. The most frequent inductions of T cells were directed against four CMV/pp65 and CMV/IE1-derived epitopes presented in HLA-A*0101, B*0702 and B*0801. To investigate whether the immunodominance of HLA-B*0702 epitopes impaired the proper induction of an HLA-A*0201-restricted response, we investigated the co-expression of HLA-B*0702 in the donors capable or incapable of mounting an HLA-A*0201-restricted immune response in our cohort finding that this was not different between the groups (50% in the non-responders and 48% in the responders). After CD137-based enrichment on day 21 of the immune responses, the frequencies of tetramer-positive cells ranged from 0.1%-21% (median, 1.9%). The antigen-specific T cells displayed oligoclonal TCR Vβ usage (*data not shown*). Due to the low level of exogenously added cytokines, no massive increases in total cell numbers were observed during the induction phase of the immune responses, resulting in a median 2.25-fold (range, 0.35-4.9) increase in total cell numbers on day 20 of the immune responses. The frequencies of $CD137^+$ T cells on day 21 after the second specific re-stimulation ranged from 0.1-3.2% (median, 1.6%). In absolute numbers, this translates into the isolation of 350 - 34×10^6 tetramer-positive cells from 1×10^6 responder cells using this procedure. This again illustrates the initial low frequencies of antigen-specific precursor cells within the total naïve responder cell populations.

To further illustrate that the Tprec frequency rather than the nature of the targeted antigen determines the success of *in-vitro* generation of a primary immune response, we generated immune responses against the HLA-A*0201-binding mHag HA-1 (n=4), LB-ADIR-1F (n=2), the HLA-B*0702-binding mHag LB-ECGF-1H (n=5), and the HLA-A*2402-binding mHag ACC1Y (n=1) from mHag-negative donors. As shown in Figure 4B, in more than 50% of the responses we were able to isolate tetramer-positive cells against these mHag.

To investigate whether the tetramer-positive T cells were high avidity T cells, we tested their capacity to recognize and kill target cells expressing endogenously processed antigen. Figure 4C shows a representative example of this analysis for a population of CMV/pp65-TPR-B*0702-specific T cells (98% tetramer-positive). The *in-vitro* generated CMV-specific T cells showed efficient cytotoxic activity in a conventional 6 h ^{51}Cr release assay against HLA-B*0702 positive EBV-LCL exogenously loaded with the 10-mer peptide, even at nanomolar concentrations. Moreover, they also recognized and lysed EBV-LCL presenting the relevant peptide after endogenous processing of either synthetic CMV/pp65 spanning overlapping peptides (pepmix), recombinant CMV/pp65 protein, or CMV/pp65 protein introduced via retroviral transduction. CMV-infected human fibroblasts were also killed by the CMV/pp65-specific T cells to levels comparable to those obtained with alloreactive T-cell clones in 6 h conventional ^{51}Cr release assays, resulting in 25-55% lysis (E:T ratio, 10:1, n=3). Figure 4D shows the reactivity of three mHag-specific T-cell clones against mHag-positive EBV-LCL, demonstrating their potent functional avidity. Overall, the cytotoxicity analyses performed with the *in-vitro* generated CMV-specific and mHag-specific T-cell populations showed that the isolated populations of tetramer-positive cells were capable of recognizing both exogenously loaded, as well as processed antigen presented by monocytes or EBV-LCL. This cytotoxic activity coincided with down-regulation of their TCR and production of interferon-γ (*data not shown*).

Isolated tetramer-positive T cells always expressed CD45RO and CD27, but the majority (90%) lacked expression of CCR-7 and CD28, illustrating their effector memory phenotype. Tetramer-positive populations could be further purified to relatively pure populations (containing >80% tetramer-positive cells) by one or two sequential rounds of enrichment using tetramer isolation (Figure 4E-F), thereby confirming the specificity of the low frequency tetramer staining directly after CD137 enrichment. Routinely, 5- to 139-fold (median, 56-fold) expansion of the isolated T cells was observed in the 10-14 days between the isolations/re-stimulations. This very marked capacity for *in-vitro* expansion in the bulk cultures and also after clonal isolation allowed the generation of large numbers of antigen-specific T cells using this procedure. Both the virus-specific T-cell clones as well as the mHag-specific T-cell clones could be maintained in culture for months and retained their proliferative and functional capacity after bi-weekly re-stimulations. We did not observe differences in the phenotype, frequencies or proliferative potential between the mHag-specific T cells and the virus-specific T cells isolated from the naïve donor repertoire after *in-vitro* stimulation.

Discussion

In this study we developed a robust method for the *in-vitro* generation of functional antigen-specific $CD8^+$ T cells from the naïve donor T-cell repertoire. In this strategy, donor PBMC were depleted of $CD45RO^+$ memory T cells and Treg and co-cultured with autologous monocyte-derived dendritic cells exogenously loaded with a mixture of relevant 9-11 mer peptides. At days 10 and 20 of the

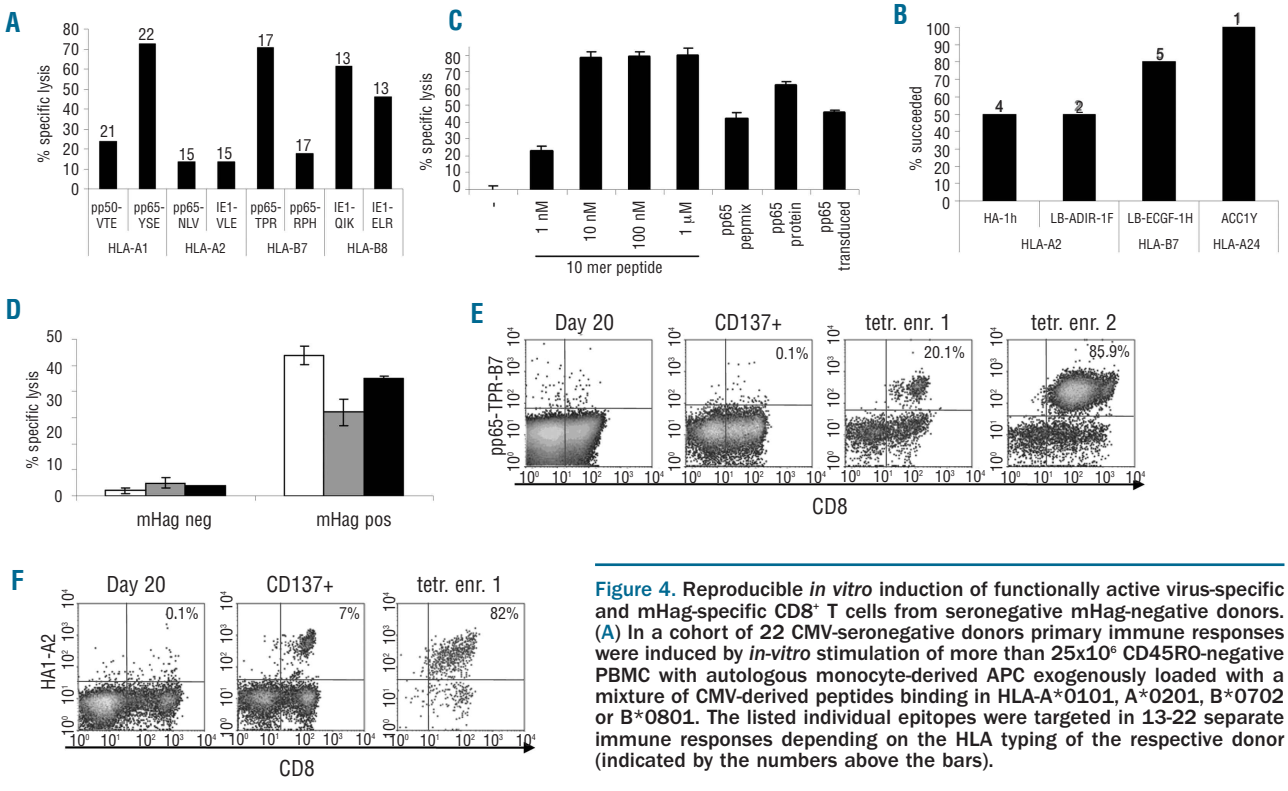

Figure 4. Reproducible *in vitro* induction of functionally active virus-specific and mHag-specific CD8⁺ T cells from seronegative mHag-negative donors. (A) In a cohort of 22 CMV-seronegative donors primary immune responses were induced by *in-vitro* stimulation of more than 25x10⁶ CD45RO-negative PBMC with autologous monocyte-derived APC exogenously loaded with a mixture of CMV-derived peptides binding in HLA-A*0101, A*0201, B*0702 or B*0801. The listed individual epitopes were targeted in 13-22 separate immune responses depending on the HLA typing of the respective donor (indicated by the numbers above the bars). The immune response was considered successful when a population of tetramer-positive cells consisting of more than 0.5% of the PBMC population was detected on day 30 of the immune response after *in-vitro* priming, expansion and CD137 enrichment. (B) In a smaller cohort of six mHag-negative donors immune responses were induced against the HLA-A*0201-restricted mHag HA-1 (n=4) and LB-ADIR-1F (n=2), the HLA-A*2402-restricted mHag ACC1Y (n=1), and the HLA-B*0702-restricted mHag LB-ECGF-1H (n=5). (C) The functional activity of the bulk populations of *in-vitro* generated CMV-specific and mHag-specific T cells was tested in conventional 6 h ^{51}Cr release assays using as target cell monocytes or EBV-LCL exogenously loaded with the respective 9- to 11-mer peptides, or presenting processed antigen. A representative example is shown for a bulk population of *in-vitro* generated HLA-B7-restricted CMV/pp65 (TPR)-specific T cells (98% tetramer-positive). HLA-B7⁺ EBV-LCL loaded with different concentrations (1 nM - 1 µM) of the specific 10-mer peptide, or presenting the relevant peptide after endogenous processing of either synthetic CMV/pp65 spanning overlapping peptides (pepmix), recombinant CMV/pp65 protein, or CMV/pp65 protein induced via retroviral transduction were used as target cells. T cells were added at am E:T ratio of 5:1. (D) The functional activity is shown for three mHag-specific T cells clones [HA-1 (□), ACC1Y (■), and LB-ECGF-1H (■)] isolated on day 20 after *in-vitro* stimulation from the naïve T-cell compartment using single-cell sorting based on tetramer expression. Cytotoxicity against mHag-negative and mHag-positive EBV-LCL expressing the relevant HLA restriction molecules (e.g. HLA-A*0201, -A*2402, and -B*0702) was tested in a conventional 6 h ^{51}Cr release assay (E:T ratio 5:1). (E-F) Tetramer-positive cell populations were further purified after initial CD137 enrichment using tetramer staining and magnetic bead selection. Depending on the initial frequencies of tetramer-positive cells, one or two sequential steps of tetramer enrichment followed by 10-day *in-vitro* expansion periods were necessary to obtain relatively pure populations containing more than 80% of tetramer-positive cells. A representative example of the frequencies of virus-specific T cells (E) and mHag-specific T cells (F) on day 20 of the immune response, 7 days after CD137 isolation, and 7 days after tetramer enrichment(s) is shown.

immune response the T cells were specifically re-stimulated with peptide-loaded autologous monocytes, and 24 hours after the second re-stimulation the responding T cells could be enriched based on their induced surface expression of the activation marker CD137. Further purification of the antigen-specific T cells was performed using specific peptide/MHC tetramers. Using this method we were able to generate functionally highly avid T cells directed against viral antigens as well as T cells directed against mHag, illustrating the broad applicability of this approach.

Although several strategies for the *in-vitro* induction of primary immune responses against defined antigens have been proposed previously,[21-25] the number of variables underlying the reproducibility of these methods limits their large scale clinical application. In this study, we investigated individual factors that together determined the likelihood of successful priming and enrichment of antigen-specific naïve Tprec. We demonstrated that depletion of CD45RO-expressing cells from the donor PBMC prior to induction of the immune response significantly increased the capacity to reproducibly induce primary immune responses, and further enrich the antigen-specific T cells. Our study explains the mechanisms underlying the improved results after CD45RO depletion. As also previously indicated by others,[22,23] depletion of CD45RO⁺ cells resulted in an increment of the frequencies of antigen-specific naïve Tprec by depletion of the memory T-cell pool. We, however, hypothesized that the resulting 2-fold increment in Tprec frequency was unlikely to be the sole cause of the major increase in efficiency of the procedure. The cytokine milieu caused by the cytokines exogenously added to the *in-vitro* cultures strongly influenced the composition of the cell cultures. Although the addition of IL-15 and IL-21 has been described to be beneficial for the expansion of antigen-specific T cells,[23,35,36] we observed massive off-target proliferation of bystander T cells and NK cells upon addition of these cytokines, hampering the efficient enrichment of the targeted T-cell populations. IL-7 as a single exogenous cytokine

supported the survival of the naïve Tprec sufficiently,[28] without inducing massive off-target background proliferation and circumvented the need for the addition of IL-2 to the culture. Since naturally occurring Treg capable of inhibiting the priming of naïve antigen-specific Tprec show high expression of the IL-2 receptor CD25, but lack expression of the IL-7 receptor CD127,[37-39] the addition of IL-7 but not IL-2 in our procedure may have favored Tprec priming.

Because most naturally occurring Treg are $CD45RO^+$,[29-31] depletion of CD45RO-expressing responder cells resulted not only in the depletion of memory T cells but also in the relative depletion of $CD4^+/CD25^{high}/FoxP3^+$ Treg. By restoring the initial Treg frequencies by adding back purified $CD4^+/CD25^{high}/FoxP3^+$ Treg to the CD45RO-depleted donor PBMC prior to the induction of the immune response, we demonstrated the capacity of the $CD45RO^+$ Treg to dampen the proper priming and/or expansion of the antigen-specific Tprec. In subsequent experiments we demonstrated that similar to the antigen-specific Tprec, the $CD45RO^+$ Treg were activated by the antigen-presenting dendritic cells used for the initial stimulation. This activation augmented their capacity to inhibit antigen-specific activation of Tprec even when they were in a numerical minority. Moreover, we demonstrated that the kinetics of Treg activation were faster than the kinetics of activation of the majority of conventional T cells. Although the absolute number of Treg within the donor PBMC population is relatively low, the extremely low frequencies of naïve antigen-specific Tprec[40] cause an imbalance in favor of the Treg locally at the site of priming of the immune response, e.g. the antigen-presenting dendritic cells. The rapid kinetics of their activation and the resulting increased capacity to impair Tprec priming may further explain the remarkable impact of Treg depletion on the capacity to induce antigen-specific primary immune responses. In memory T-cell responses Treg-mediated suppression is less likely to occur since the frequencies of antigen-specific memory cells may override the number of Treg and their priming does not require interaction with professional APC.

Using this procedure, we were able to reproducibly induce CD8 T cells against multiple viral antigens as well as mHag only when the responses were initiated from more than 25×10^6 CD45RO-negative donor PBMC, illustrating that the Tprec frequencies rather than the nature of the targeted antigen determines the likelihood of successful induction and expansion of the antigen-specific T cells. All isolated antigen-specific T-cell populations showed a high avidity reaction pattern, as demonstrated by their capacity to efficiently recognize and lyse target cells presenting endogenously processed antigen. The CD137 enrichment step in our procedure may further enhance the proliferative and survival capacity of the antigen-specific T cells due to the beneficial co-stimulatory signal supplied by cross-linking of CD137 on the T-cell surface during the isolation procedure.[41,42] This procedure may allow the generation of antigen-specific T cells for clinical application. However, since the frequencies of Tprec against single antigens is low, further enrichment and vigorous post-isolation expansion remain necessary to allow *in-vivo* infusion of substantial numbers of specific cells and limited numbers of T cells of unknown specificity. Depending on frequencies of tetramer-positive cells in the T-cell population after CD137 enrichment, one or two sequential enrichment steps based on tetramer isolation were necessary to obtain a T-cell product containing more than 80% tetramer-positive cells, suitable for clinical application. This would require a 30-40 day culture period. In the experiments described in this article, post-isolation expansion was performed using allogeneic feeder cells and PHA, which is not suitable for the production of cells for clinical application. In a selected number of experiments we demonstrated similar expansion of the cells using autologous feeder cells, a medium containing IL-7 and IL-15, and clinical grade CD3/CD28 microbeads (*data not shown*), allowing the generation of antigen-specific T cells from the naïve donor repertoire under Good Manufacturing Practice conditions. Although this method is still complex and laborious, these cells may be of great therapeutic importance in selected patients. A further increment of the antigen-specific Tprec frequencies by increasing the number of targeted antigens using a cocktail of multiple peptides as an '*in-vitro* vaccine' may further increase the robustness of the procedure. Since CD137 is expressed on activated CD4 T cells as well,[19] the method can also be applied for the isolation of T cells targeting HLA-class II-restricted antigens.

In conclusion, we present a robust method for the *in-vitro* induction and isolation of antigen-specific T cells from the naïve repertoire. We demonstrated that the likelihood of successful generation of primary immune responses is determined by a delicate balance between the numbers of naïve antigen-specific Tprec and the numbers and activation state of Treg locally at the site of priming of the immune response.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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