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

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Online Supplementary Appendix

Design and Methods

Selection of cell populations

Monocytes were isolated from donor peripheral blood mononuclear cells (PBMC) using magnetic CD14 cliniMACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. To transform these monocytes into professional antigen-presenting cells (APC), the cells were seeded at a concentration of 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (IMDM, BioWhittaker, Lonza, Verviers, Belgium) containing 10% irradiated prescreened human serum, and cultured for 2 days in the presence of 100 ng/mL granulocyte-monocyte colony-stimulating factor (GM-CSF; Novartis, Basel, Switzerland), and 500 IU/mL interleukin (IL)-4 (kindly provided by Schering-Plough, Innishammon, Cork, Ireland). Subsequently, the cells were matured for 2 days in the presence of 100 ng/mL GM-CSF, 10 ng/mL IL-1β (Cellgenix, Freiburg, Germany), 10 ng/mL IL-6 (Cellgenix), 10 ng/mL tumor necrosis factor-α (Boehringer Ingelheim, Alkmaar, The Netherlands), 500 IU/mL interferon-γ (Immukine; Boehringer Ingelheim), and 1 μg/mL prostaglandin E2 (PGE_2; Sigma Aldrich, Zwijndrecht, The Netherlands).[43,44]

For the analysis of T-cell activation kinetics, naïve and memory T-cell populations were selected by negative selection of T cells using the pan T-cell isolation kit II (Miltenyi Biotec), followed by positive selection of $CD45RO^+$ memory T cells or $CD45RA^+$ naïve T cells using CD45RO or CD45RA MACS isolation beads (Miltenyi Biotec), respectively.

Naturally occurring regulatory T cells (Treg) were enriched by magnetic bead separation using CD4 and CD25 microbeads (Miltenyi Biotec) and/or flowcytometric cell sorting of $CD4^+/CD25^{high}/CD127^-$ cells. Purity was confirmed by FoxP3 staining.

In the initial induction experiments, CD14-depleted donor PBMC were used as responder cells. In the large cohort, subsequent depletion of $CD45RO^+$ cells was performed using CD45RO magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions.

Peptides and tetramers

The HLA-A*0101-binding peptides CMV/pp50 VTEHDTLLY, CMV/pp65 YSEHPTFTSQY, HLA-A*0201-binding peptides CMV/pp65 NLVPMVATV, CMV/IE1 VLEETSVML, mHag/HA-1 VLHDDLLEA, mHag/LB-ADIR-1F SVAPALALFPA, HLA-A*2402-binding peptide mHag/ACC1Y DYLQYVLQI, HLA-B*0702-binding peptides CMV/pp65 TPRVTGGGAM, CMV/pp65 RPHERNSFTVL and mHag/LB-ECGF-1H RPHAIRRPLAL, and the HLA-B*0801-binding peptides CMV/IE1 QIKVRVDMV and ELRRKMMYM were synthesized using standard solid-phase strategies (Department of Immunohematology, Leiden University Medical Center, Leiden, The Netherlands) and used for stimulation of antigen-specific T cells. CMV/IE1 and CMV/pp65 protein-spanning overlapping peptide pools were purchased from JPT Peptide Technologies GmbH (Berlin, Germany).

Allophycocyanin and phycoerythrin (PE)-labeled peptide/MHC complexes (tetramers) were generated within our own department according to the methodology described previously.[1]

Flow cytometry analysis

Immunophenotype analysis of the monocyte-derived APC was performed by staining the cells for 30 min at 4°C with fluorescein isothiocyanate (FITC)-labeled CD1a (BD Pharmingen, Breda, the Netherlands), CD40 (Serotec, Oxford, UK), CD86 (BD Pharmingen), and HLA-ABC (w6.32, serotec) monoclonal antibodies, and PE-labeled CD11c (BD), CD80 (BD), and CD83 (Sanbio, Uden, the Netherlands) monoclonal antibodies.

The composition of the responder cell populations was determined by staining the cells for 30 min at 4°C with FITC-labeled CD45RO (Caltag/Invitrogen, Breda, The Netherlands), CD62L (Bender MedSystems/eBioscience, Huissen, The Netherlands), TCR-αβ and TCR-γδ (BD Pharmingen) monoclonal antibodies, PE-labeled CD45RA (Beckman Coulter, Woerden, the Netherlands), CD27 (BD/Pharmingen), CD4 (Caltag/Invitrogen) and CD56 (BD Pharmingen) monoclonal antibodies, peridinin-chlorophyl (PerCP)-labeled CD8 (BD Pharmingen) monoclonal antibody, and allophycocyanin-labeled CD3 (BD Pharmingen) monoclonal antibodies.

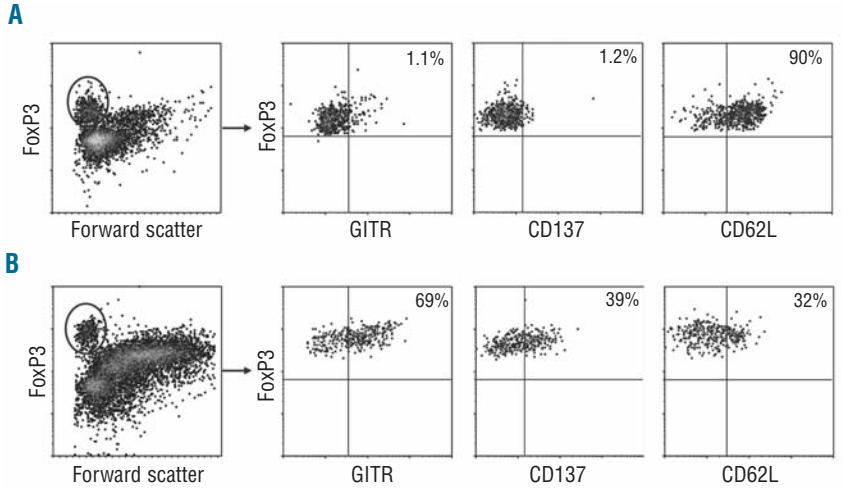
Antigen-specific T cells were stained with allophycocyanin- and PE-labeled tetramers for 15 min at 37°C, followed by CD4/CD8 counterstaining for 15 min at 4°C.

Intracellular FoxP3 staining was performed using the FoxP3 staining kit (FoxP3-APC monoclonal antibody, clone PCH-101, eBioscience, Huissen, the Netherlands) according to the manufacturer's instructions.

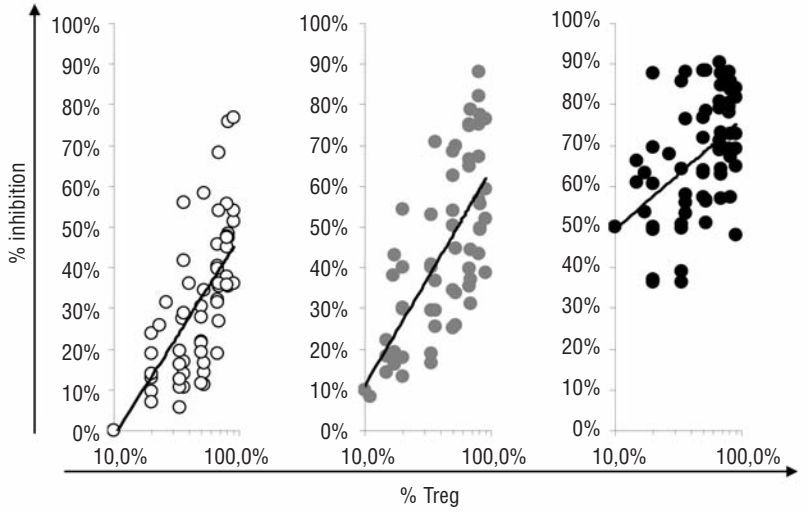
Cells were analyzed using FACS Calibur, Cellquest software (BD), and FlowJo software (Tree Star, Ashland, OR, USA).

Proliferation/inhibition assays

The inhibitory potential of isolated Treg was determined using *in-vitro* proliferation assays. Responder T cells were labeled with the fluorescent dye PKH26 (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's instructions, washed, and incubated in culture medium overnight. PKH26-labeled responder cells were seeded at a density of 25,000/well in 96-well plates in T-cell medium supplemented with 50 IU/mL IL-2 (Chiron, Amsterdam, The Netherlands). Proliferation was induced using either CD3/CD28 expansion beads (Invitrogen) or by the induction of a mixed lymphocyte reaction (MLR) using irradiated HLA-mismatched allogeneic EBV-LCL as stimulator cells (R:S ratio, 1:1) in the presence or absence of different numbers of purified Treg. After 5 days, samples were counterstained with CD4/CD8 antibodies. The wells were harvested, a fixed amount of fluorescent microspheres was added (Flowcount beads; Beckman Coulter, Mijdrecht, The Netherlands) to allow quantitative analysis, and dead cells were excluded using low-dose propidium iodide (PI) (0.2 μg/mL). The cells were directly analyzed using a FACS Calibur and Cellquest software (BD). Absolute numbers of non-dividing/non-activated ($PKH^{bright}/CD69^{-}$) T cells were quantified. The percentage of responding T cells was calculated as follows: 100-[100x(absolute number of non-activated/non-dividing T cells after stimulation / absolute number of non-activated/non-dividing T cells without stimulation)]. The percentage of inhibition was calculated using the formula: 100-[100x(percentage of responding T cells in the presence of Treg / percentage of responding T cells in the absence of Treg)].



Online Supplementary Figure S1. Activation of naturally occurring FoxP3-positive regulatory T cells by autologous monocyte-derived APC. The activation state of naturally occurring $CD4^{+}/FoxP3^{+}$ Treg within the total PBMC population under steady state conditions (A) and after 24 h of co-culture with autologous monocyte-derived dendritic cells (B) was determined by flow cytometric analysis of co-expression of GITR, CD137 or CD62L (n=3). Treg were distinguished from activated conventional T cells using a forward scatter/FoxP3 gating strategy demonstrated in the left dot plots.



Online Supplementary Figure S2. Effect of activation of regulatory T cells on their inhibitory potential. $CD4^{+}/CD25^{high}/FoxP3^{+}$ Treg were isolated by magnetic bead separation (MACS) and used in a suppression assay. PKH26-labeled CD25-negative responder T cells (Tresp) from healthy donors were stimulated with CD3/CD28 expansion beads in the absence or presence of different frequencies of Treg (50% Treg indicates a Tresp:Treg ratio of 1:1). The percentage of responding T cells on day 5 after CD3/CD28 stimulation was measured by quantitative flow cytometric analysis using proliferation and CD69 expression as markers for responding T cells. The percentage inhibition reflects the decline in the number of T cells responding to CD3/CD28 stimulation in the presence of Treg. The inhibitory capacity of freshly isolated Treg (○), Treg activated for 48 h by autologous APC (●), and Treg activated by 48 h CD3/CD28 stimulation (●) is shown. Treg activation by APC or CD3/CD28 stimulation significantly improved their inhibitory potential (P=0.03 and P<0.01, respectively).