Supplemental Data

Detailed Materials and Methods for Clinical scale DC production and co-culture with $CD25^+$ cells

Mononuclear cells for production of DC were collected from intended recipients by apheresis. Approximately 4 total body blood volumes were leukopheresed. Resultant product was further enriched for mononuclear cells by density gradient separation over 1.077 gm/mL Ficoll. Cells were washed and resuspended in PlasmaLyteA infusion solution supplemented with 0.25% human albumin and 10% DMSO, then stored in vapor phase of liquid nitrogen. Six days prior to start of co-culture with $CD25^+$ cells, the DC precursor cells were thawed, washed, and re-suspended in CellGenix DC medium. $3x10^8$ cells each were aliquoted into $225cm^2$ tissue culture flasks and placed in a 37°C incubator for three hours. Flasks were gently rocked, and all nonadherent cells were removed. Flasks were charged with 30 mL of CellGenixDC medium, supplemented with 1 μg/mL GM-CSF and 0.5 μg/mL IL-4 and were incubated at 37°C in a 5% CO_2 humidified incubator for six days, with addition of an additional 1 μg/mL GM-CSF and 0.5 μg/mL IL-4 on the third day of culture. At the conclusion of incubation, DC were removed from the flasks and washed in RPMI1640 medium. The re-suspended DC were irradiated (30 Gy) using a ^{137}Cs source to prevent possible replication of contaminating tumor cells.

To initiate the co-cultures, $CD25^+$ cells, which were held in liquid nitrogen storage following purification, were thawed, washed and resuspended in RPMI1640 medium. DC and $CD25^+$ cells were then admixed at a ratio between 2:1 and 1:2. RPMI 1640 medium supplemented with 10% heat inactivated human AB positive serum; 10 U/mL IL-2; 10 ng/mL IL-15; 100 ng/mL rapamycin was added to adjust the final concentration of $CD25^+$ cells to $2x10^6$ cells per mL, and 60 mL of this suspension was added to each $225cm^2$ tissue culture flask. Flasks were placed in incubation at 37°C for six days, at which time an additional 40 mL of AB serum, IL-2, IL-15 and rapamycin supplemented medium was added on days 6, 8 and 10. Final harvest followed an additional six days of incubation.

Supplemental methods for Foxp3 TSDR demethylation analysis by quantitative PCR:

Methylated or demethylated Foxp3 TSDR were cloned into pCR2.1-Topo vector (Life Technologies), used to create standard curves. Briefly, PCR products were amplified by using methylated or demethylated specific TSDR forward and reverse primers from bisulphate-treated genomic DNA of sorted naïve Treg. DNA fragments were then cloned into pCR2.1-Topo vector and sequences were checked by DNA sequencing. The following methylated and demethylated specific TSDR primers and probes were used for PCR; Methylated forward 5'-GTTTTCGATTTGTTTAGATTTTTTCGTT-3', reverse: 5'-CCTCTTCTCTTCCTCCGTAATATCG-3', hydrolysis probe: 5'-TAMRA-ATGGCGGTCGGATGCGTCGGGT-FAM-3' and demethylated forward 5'-GTTTTTGATTTGTTTAGATTTTTTTGTT-3', reverse 5'-CCTCTTCTCTTCCTCCATAATATCA-3', hydrolysis probe, 5'-TAMRA-ATGGTGGTTGGATGTGTTGGGT-FAM-3'. Standards were diluted at a concentration of 2E7, 2E6, 2E5, 2E4 and 2E3 plasmid copies of each methylated or demethylated TSDR. Genomic DNA was isolated using the DNeasy Blood and tissue kit (Qiagen) from sorted naïve or expanded Treg. About 200 to 500 ng of genomic DNA was treated with bisulphite using EZ DNA Methylation-Gold™ Kit (Zymoresearch). After bisulphite conversion, qPCR was performed with a final volume of 20 µl using TaqMan® Universal PCR Master Mix No AmpErase® UNG (Life Technologies) containing 5µM each of methylation or demethylation TSDR specific forward or reverse primers, 10µM of methylation or demethylation TSDR specific hydrolysis probe and 20 ng of bisulphite-treated genomic DNA. PCR conditions consisted of a 95^0C preheating step for 10 min and 50 cycles of 95^0C for 15s followed by 1 min at 61^0C.

Supplemental methods for the generation of mHA-specific Treg clones:

Treg clones were generated from a female donor stimulated by irradiated DC from her HLA-identical brother. On day 6, culture supernatant was collected from each well and measured for TGF-β by ELISA (ebiosciences). Positive wells were pooled and Treg were cloned by limiting dilution (3, 1 or 0.3 cells/well) in 96-well, round-bottomed plates in the presence of irradiated (3000 cGy) sibling male mononuclear cells, IL-2 (10 U/ml), IL-15 (10 ng/ml) and rapamycin (100 ng/ml). Split well assay was performed on day 14 of cloning to test for specificity, and Treg clones were selected based on higher TGF-β release in response to the male sibling compared to self DC. LCL derived from the female or male blood cells were used as stimulators in some experiments. Clones were expanded using male sibling LCL, allogeneic feeders and cytokines before cryopreservation. Tconv clones from the same donor were established with a similar protocol, except for stimulation with IL-2 as sole cytokine, and assay for TNF release. The H-Y associated genes, DBY, UTY, SMCY or DFFRY-2 encoded by the Y chromosome, were cloned into a Moloney murine Leukemia virus-based retroviral vector LZRS with GFP tag and kindly provided by M. Griffioen and J. H. Falkerburg (Leiden University Medical Center, Netherlands). Phoenix-A packaging cells were obtained from SM Sebti (Moffitt Cancer Center) and transfection were performed as described [1]. DBY, UTY, SMCY or DFFRY-2-encoded retroviral vectors were transfected each into Phoenix-A cells using the X- tremeGene 9 DNA transfection reagent (Roche) and, after two days, transfectants were selected with 2 μg/ml of puromycin (Millipore) for 14 days. After 14 days, about 6 millions Phoenix-A cells that were transfected and puromycin selected were replated in 10-cm Petri dish in 10 ml of DMEM supplemented with 10% FBS without puromycin. After 24 h, retroviral supernatant was collected and used for transducing LCL [1]. DBY, UTY, SMCY or DFFRY-2 genes were retroviral transduced into female LCL using RetroNectin-bound Virus (RBV) infection method. Briefly, 20 μg/ml of RetroNectin was coated on to 24 well tissue culture plates for overnight; retroviral supernatant was added to

the well for 6 h and washed. About 1 x 10^5 female LCL was incubated to the RetroNectin coated plates in 37^0C incubator under 5% CO_2 for 2 days. The transduction efficiency was measured by the expression of the GFP marker, and cells were FACS-sorted based on their GFP Positivity. To assign specificity, Treg or Tconv clones were cultured with the DBY, UTY, SMCY, DFFRY-2 or empty vector-transduced self LCL (2 x 10^4) in the presence of cytokines with proliferation measured after 72h. To determine the specificity of suppression, Treg clones were titrated with fixed numbers of Tconv clones of known mHA specificity stimulated by the LCL expressing the appropriate mHA.

Additional Reference:

1. Vogt MH, van den Muijsenberg JW, Goulmy E, et al. The DBY gene codes for an HLA-DQ5-restricted human male-specific minor histocompatibility antigen involved in graft-versus-host disease. *Blood*. 2002;99(8):3027-3032.