Supplement.

Materials and Methods.

Antibodies and Reagents.

The fluorochrome conjugated and unconjugated anti-human α-smooth muscle actin (α-SMA, clone 1A4) murine monoclonal antibodies (Abs) were purchased from Sigma (St Louis, MO). Fluorochrome conjugated forms of $IgG1_{\kappa}$, IgG_{2a}, isotype controls and monoclonal antibodies directed against human CD90 (clone 5E10) were purchased from BD PharMingen (San Diego, CA). Appropriate fluorochrome conjugated murine and rat isotypes as well as monoclonal antibodies directed against human CD4 (clone RPA-T4), CD127 (clone eBioRDR5), CTLA4 (clone 14D3) and FoxP3 (clone PCH101) were purchased from eBioscience (San Diego, CA). Appropriate fluorochrome conjugated monoclonal antibodies directed against human CD25 (clone M-A251) were purchased from BD Pharminge. Alexa Fluor® (AF®) mAbs labeling kits were purchased from Invitrogen (Carlsbad, CA). PGE_2 has been purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

Generation of human DCs from peripheral blood mononuclear cells.

DCs were generated from human peripheral blood via a standard protocol. Briefly, PBMCs were obtained from a buffy coat preparation using Ficoll-Paque™ Plus (Amersham Bioscience, Piscataway, NJ) density gradient centrifugation and plastic adherence. Adherent cells wererecovered with ice-cold 0.2 mM EDTA/PBS. The cells were resuspended at a density of $1x10^6$ cells/ml in complete RPMI-10 containing 800 U/ml GM-CSF (eBioscience, San Diego, CA) and 500 U/ml IL-4 (eBioscience). Fresh media and cytokines were provided to the cultures each 3 days. On day 8 or 9, the cells were recovered, characterized by flow cytometry for the expression of CD83 and CD11c and used in co-culture experiments.

Real Time RT-PCR.

The total cellular RNA was isolated from T cells using RNeasy® RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RT real-time PCR was performed according the Applied Biosystems's two-step RT real time PCR protocol (Applied Biosystems, Foster City, CA). The assays-on-demand™ gene expression assay mix (Applied Biosystem) for human 18S, FoxP3, IL-10 or TGF-β1,TGF-β2, or TGF-β3 (a 20X mix of Unlabeled PCR primers and TaqMan® MGB probe, FAM™ dye-labeled) and 2 µL of cDNA were added to the PCR reaction mix. The reaction was carried out in 20 µL final volume using

GeneAmp® 5700 Sequence Detection System (Applied Biosystems) according to the following protocol: 2 min at 50°C, 10 min at 95°C (1 cycle) and 15 sec 95°C and one min at 60°C (40 cycles). The negative controls were included in the RT real time two-step reaction. The endpoint used in real-time PCR quantification, C_T, defined as the PCR cycle number that crosses the signal threshold. C_T values range from 0 to 40, with the latter number assumed to represent no product formation. Quantification of cytokine gene expression was performed using the comparative C_T method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to the human housekeeping gene, 18S rRNA. In order to calculate the fold change (increase or decrease), the C_T value of 18S rRNA was subtracted from C_T value of the target cytokine gene to yield the ΔC_T. Change in the expression of the normalized target gene as a result of experimental conditions was expressed as $2^{-\Delta\Delta C}T$ where $\Delta\Delta C_T = \Delta C_T$ experimental samples - ΔC_T biological control.

CFSE proliferation assays

Proliferation of $CD4^+CD25^{high}FoxP3^+$ T cells in cocultures with CMFs was assessed using the cell trace™ CFSE cell proliferation kit (Molecular Probes, Inc., Eugene, OR). Membrane labeling of T cells with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) according to the manufacturer's instructions. CFSE-labeled T cells were then cocultured with CMFs (at a ratio of 10:1, respectively) for a maximum of 7 days. T cells were then recovered from co-culture by washing with Cell Dissociation buffer (Sigma), and immunostained for the markers of interest. T-cell proliferation was assessed by flow cytometry and analyzed using FACSDiva 6.0 software (Becton Dickenson) and FlowJo (Tree Star, USA).

Flow cytometry

T cells were stained for surface and/or intracellular markers according to each experiment. Single- and multi- color immunostaining was performed according to standard surface and intracellular FACS staining BD PharMingen protocols. Cells were washed between and after each staining and fixed with 1% paraformaldehyde in PBS, and analyzed by flow cytometry in FACSCanto™, FACSAria™, and FACScan cytometers (BD Biosciences). The FACS analysis was carried out with optimal compensation set for green, yellow, far-red fluorescences. Percentage of cells that were antibody-positive was calculated by comparison with the corresponding isotype control. Flow cytometry data were analyzed by using Flow Jo (Tree Star Inc., Ashland, OR) and BD FACS Diva (BD Biosciences) software.

T Cell Suppression Assays

$CD4^+$ T cells recovered from the co-cultures with CMFs were washed twice with HANKS solution without Ca^{2+} and Mg^{2+} (Cellgro, Herndon, VA) and re-suspended in standard culture media at concentration 10^7 cells/mL. The population of $CD4^+CD25^{high}$ cells were sorted by staining with monoclonal antibodies against CD4 and CD25 (BD Biosciences) and sorted into $CD4^+CD25^{bright}$ and $CD4^+CD25^-$ cells using the BD FACSAria cell sorting system (Becton Dickinson, Heidelberg, Germany). The purity of the $CD4^+CD25^{high}$ T cells after sorting was >98% (as controlled by flow cytometry). For T cell suppression assays, 10^5 naive $CD4^+$ T cells isolated from PBMC, as described above, were activated by using T cell Activation/Expansion kit (Miltenyi Biotec) according to the manufacturer's instructions. The activated T cells were cultured in the absence or presence of increasing numbers the newly generated $CD4^+CD25^{bright}$ T cells at various responder:suppressor ratios. Cells were cultured for 72 h at 37°C in 5% CO_2 by using 96-well flat bottom plates. One µCi of [^{3}H]-thymidine was added 18 hours prior to cell harvesting. At the end of the cultures, cells were harvested onto glass fiber filter mats and [^{3}H]-thymidine incorporation (c.p.m.) was determined using a liquid scintillation counter (PerkinElmer, Wellesley, MA). The direct suppressive effect of $CD4^+CD25^{high}$ T cells was evaluated by measuring [^{3}H]thymidine incorporation. Results were expressed in c.p.m. as the mean c.p.m.± SE of triplicate cultures.

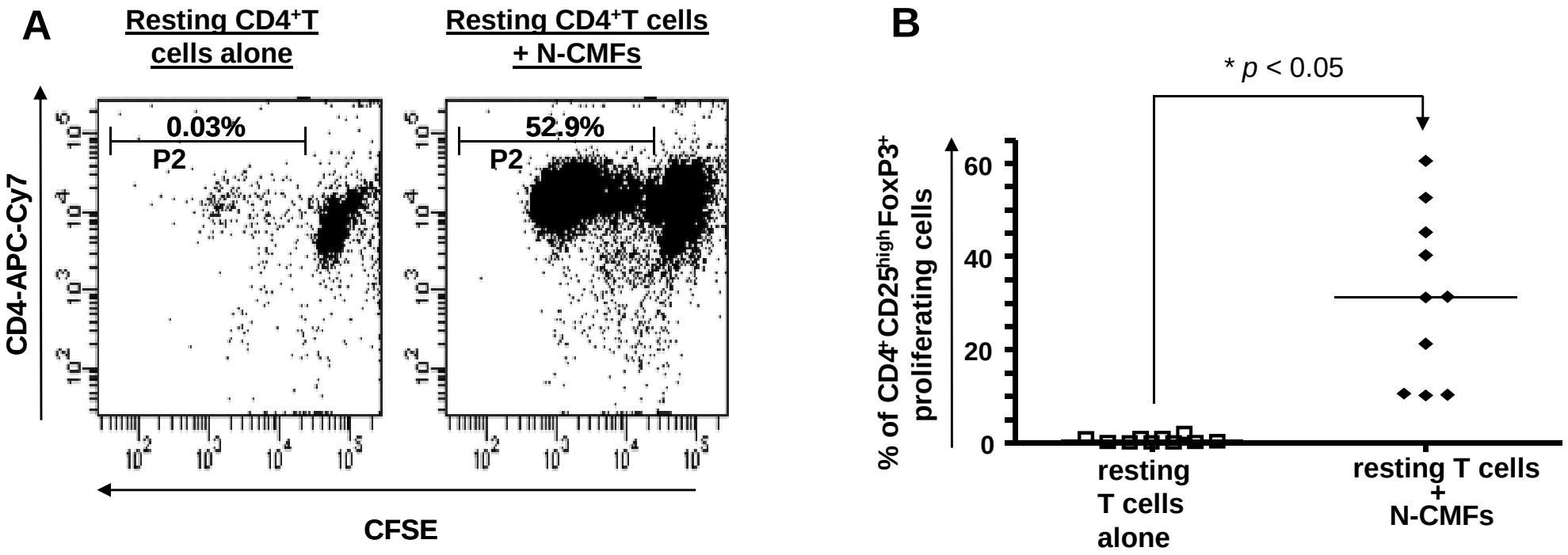

Figure S1. $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells present in the proliferating fraction of $CD4^+$ resting T cells primed by normal (N-) CMFs. CFSE-labeled resting $CD4^+$ T cells were cultured without or with allogeneic N-CMFs at a ratio 1:10 for 7 days in 24 well plates. Co-cultured cells were washed from plates and subjected to surface CD4 and CD25 and intracellular FoxP3 staining. (**A**) Proliferating $CD4^+$ T cells derived from monoculture or co-culture with N-CMFs are marked in gate P2. (**B**) Proliferation of T cells with regulatory phenotype ($CD4^+$ $CD25^{high}$ $FoxP3^+$) from resting $CD4^+$ T cells primed by allogeneic N-CMFs. Percentage of the dividing $CD4^+$ $CD25^{high}$ $FoxP3^+$ T cells in response to the N-CMF allogeneic stimulation was measured. Results are calculated as the mean value of the percentage of the dividing cells for five independent allogeneic pair of CMFs and CD4+ T cells healthy donors ± SD. Each assay was conducted in duplicate. * $p < 0.05$.

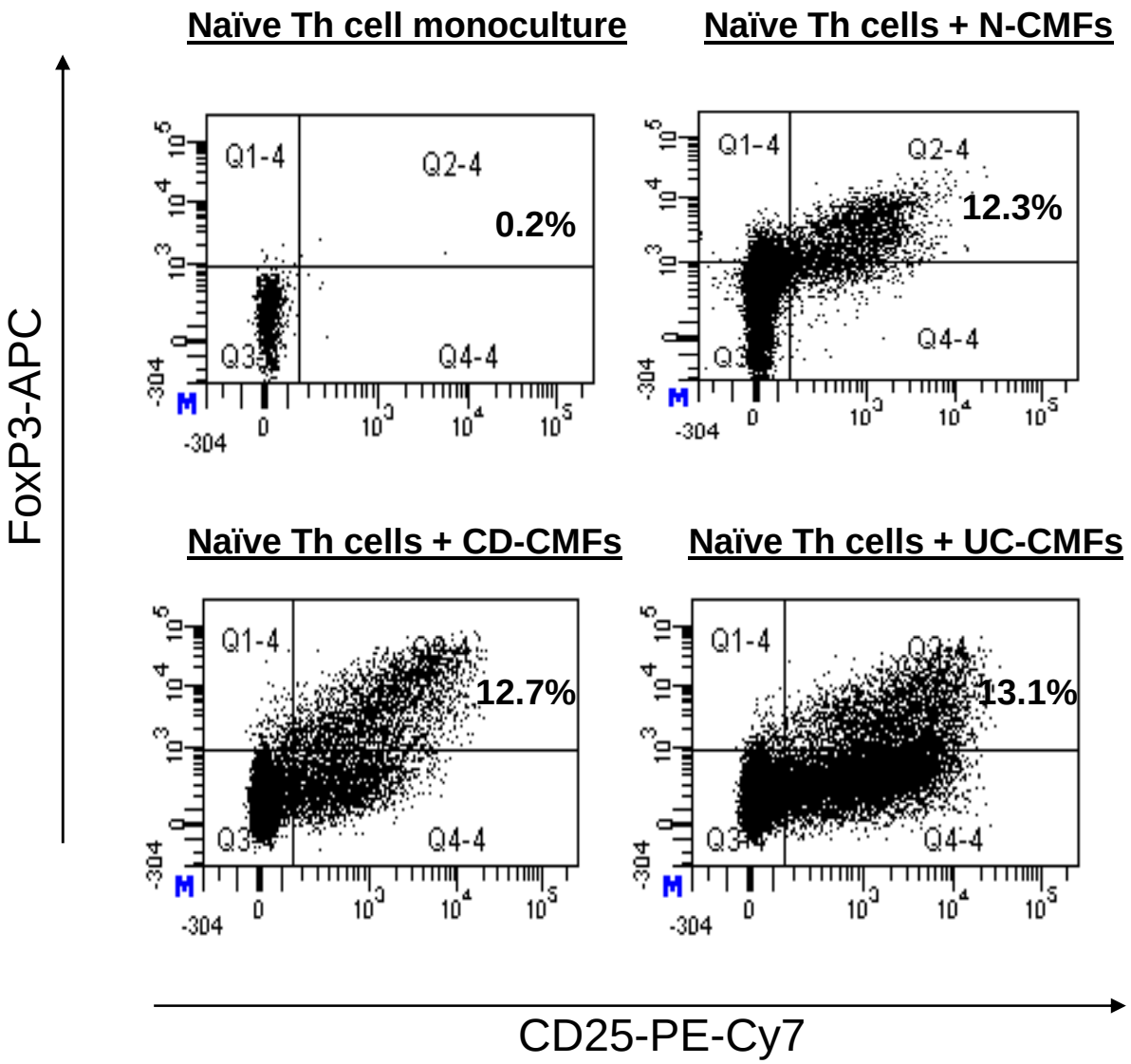

Figure S2. Induction of the CD4+CD25^{high}FoxP3+ T cells from naïve CD4^{+}CD45RA^{+} T cells (Th_0) by allogeneic N-, CD- and UC-CMFs. The surface CD25 and intracellular FoxP3 expression in the CD4+ T cells primed by allogeneic N-, Cd- and UC-CMFs analyzed using flow cytometry. Live events were gated and then CD4+ T cells were gated (in order to eliminate contamination of the analyzed population with CMFs) and analyzed for CD25 and FoxP3 expression. The appropriate isotype controls were included in the experiments. A representative experiment is shown (n=9).