Patients and controls

After informed consent was obtained from all patients, using protocols approved by the Institutional Review Board of the National Heart, Lung and Blood Institute of Health, heparinized peripheral blood samples were obtained from 20 patients (age range: 13-52 years) who were treated in Hematology Branch clinics (Supplemental Table 1). The diagnosis of aplastic anemia was based on the criteria of the International Agranulocytosis and Aplastic Anemia Study. Fourteen healthy volunteers served as controls (age range: 18 to 55 years). Marrow cytogenetics were normal in all clinical cases and Fanconi anemia was excluded by chromosome breakage studies in children and younger adults. Thirteen patients (patient # 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 17, and 20) were re-examined for Treg and FOXP3 expression 3-6 months after the first sampling.

Antibodies and immunoblots

Anti-FOXP3 monoclonal antibody (mAb) and anti-TLR2 Ab were purchased from BioLegend (San Diego, CA) and anti-NFAT1 Ab from BD Pharmingen (San Jose, CA). The HRP- conjugated secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Cytoplasmic and nuclear extracts from purified CD4+ CD25+ T cells were prepared using the CD4+ CD25+ T cell isolation kit (Miltenyi Biotech, Auburn, CA) and examined in immunoblot experiments as previously described[11]. The amount of antibodies used for CD4, CD25, and FOXP3 staining was determined by titration in preliminary experiments to be at saturated doses. The purified populations were

analyzed for purity by flow cytometry; all the samples were approximately 85-90% CD4+ CD25+ or CD4+ CD25- as examined by flow cytometry (supplement Figure 1A).

Confocal microscopy

Purified CD4+CD25+ T cells from patients and controls were analyzed for NFAT1 and FOXP3 expression by confocal microscopy[17]. Cells were fixed on glass cover slips previously coated with poly-L-lysine (Sigma) and stained with anti-FOXP3 and anti-NFAT1 antibodies, followed by secondary FITC- and Texas-Red -conjugated secondary Abs, respectively (Jackson Immuno-Research Laboratories, PA). Immunofluorescence was examined by confocal laser microscopy with Zeiss 510 confocal system equipped with UV-Vis lasers (Carl Zeiss Inc, Jena, Germany). Images were acquired sequentially using a 488-nm laser line and emission between 505-580nm for FITC and differential interference contrast (DIC) and a 561-nm laser line and emission over 585-nm for Texas Red. DAPI was used for nuclear staining because it forms complexes with natural double-stranded DNA. High-resolution (100nm/pixel) images were obtained with a 63x, water immersion objective and deconvolved using Huygens software (SVI, Hilversum, Netherlands), and assembled using Imaris 5.0 software (Bitplane AG, Switzerland).

T cell transfections and quantitative cell fluorescence analysis

Purified CD4+ CD25+ and CD4+ CD25- T cells from patients and healthy donors were transiently transfected[18, 19] with a GFP-wild-type NFAT1 plasmid[20] (kind gift of Dr A. Rao, Harvard University, Cambridge, MA) using the Human T cell Nucleofector Kit

from Amaxa Biosystems (Amaxa Inc, Gaithersburg, MD), based on manufacturer's instructions. Cells were left overnight in medium supplemented with 10% FCS at 37^0 C, and 18hrs later were stimulated with PMA and ionomycin. Twenty-four hrs later, cells were collected and stained with FOXP3 antibody and examined under confocal microscopy, and quantitative analysis of cell fluorescence by confocal microscopy was performed. Transfection efficiency was determined by GFP expression. In order to obtain valuable quantitative data, images were acquired using identical settings of the instrument that avoid saturation of the brightest pixels. Series of 12-bits images along the z-axis were obtained throughout the cells with a 63x, 1.2-numerical-aperture C-Apochromat water immersion objective. The integrated sum of total fluorescence though all optical sections in the 3-D series was calculated and compared among the CD4+ CD25+ and CD4+ CD25- transfected T cells. 2-D projections (using maximum-pixel-intensity algorithm) of the 3-D fluorescence were generated. FOXP3 fluorescence intensities were color-coded using a look-up-table with a 0-4095 range to create pseudocolor-mapped images.

Quantitative determination of IL-2

Purified CD4+CD25+, CD4+CD25-, and NFAT1-transfected CD4+CD25- T cells from healthy volunteers were stimulated with aCD3 mAb for 24 hrs or left without treatment. IL-2 secretion was measured in culture supernatants by ELISA based on manufacturer's instructions (R&D Systems, Minneapolis, MN).

NFAT1-siRNA

Purified CD4+ CD25+ T cells from two healthy controls were used for NFAT1-siRNA experiments using an NFAT1 si-RNA and a control-siRNA, based on manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were harvested 72 hrs later; extracts from these cells were examined by immunoblot for NFAT1 and FOXP3 expression.