Regulatory T cells are believed to control the development and progression of autoimmunity by suppressing autoreactive T cells. Decreased numbers of CD4+CD25+ FOXP3+ T cells (Tregs) are associated with impaired immune homeostasis and development of autoimmune diseases. The transcription factors FOXP3 and NFAT1 have key roles in regulatory T-cell development and function. We show that Tregs are decreased at presentation in almost all patients with aplastic anemia; FOXP3 protein and mRNA levels also are significantly lower in patients with aplastic anemia and NFAT1 protein levels are decreased or absent. Transfection of FOXP3-deficient CD4+CD25+ T cells from patients with a plasmid encoding wild-type NFAT1 resulted in increased FOXP3 expression in these cells. By NFAT1 knockdown in CD4+CD25+ T cells, FOXP3 expression was decreased when NFAT1 expression was decreased. Our findings indicate that decreased NFAT1 could explain low FOXP3 expression and diminished Treg frequency in aplastic anemia. Treg defects are now implicated in autoimmune marrow failure. (Blood. 2007;110:1603-1606)

Lymphocyte isolation, flow cytometry, and immunobots
CD4, CD25, and FOXP3 expression was examined in peripheral blood mononuclear cells (PBMC) by 3-color flow cytometry as previously described11 using an APC-antihuman FOXP3 staining kit (eBioscience, San Diego, CA).13 Immunoblot experiments were performed as previously described11 (Document S1).

Quantitative real-time polymerase chain reaction
FOXP3 gene expression was measured in CD4+CD25+ T cells as previously described.15 All polymerase chain reaction assays were performed in duplicate and reported as the mean.

Confocal microscopy and T-cell transfections
NFAT1 and FOXP3 expression was examined by confocal microscopy as previously described17 (Document S1). Transfections were performed18,19 using a GFP-wild-type NFAT1 plasmid20 (a gift from Dr. A. Rao, Harvard University, Cambridge, MA) and examined by confocal laser microscopy with Zeiss 510 confocal system equipped with UV_VIS lasers (Carl Zeiss, Jena, Germany). High-resolution images were obtained with a 63 ×, water emission objective and deconvolved using Huygens Software (SVI, Hilversun, the Netherlands), and assembled using Imaris 5.0 software (Bitplane AG, Zurich, Switzerland) (Document S1). NFAT1-siRNA was performed in CD4+CD25+ T cells based on the manufacturer’s instructions. Interleukin-2 secretion was measured in culture supernatants by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).


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Densitometry and statistics

Densitometry of bands of interest was performed as previously described.11 Statistical analysis was performed using the $t$ test (Prism Software, San Diego, CA); $P$ values of less than .05 were considered statistically significant.

Results and discussion

Decreased circulating regulatory T-cell number in patients with acquired aplastic anemia

We first measured the frequency of Tregs in aplastic anemia (results are $\pm$ SEM). All patients had significantly decreased numbers of CD4$^{+}$CD25hi$^{-}$ T cells compared with healthy donors ($0.08\% \pm 0.01\%$ versus $0.46\% \pm 0.1\%, P < .001$, Figure 1A,B). The frequency of CD4$^{+}$CD25hi$^{-}$FOXP3$^{+}$ T cells in patients also was much decreased compared with that from the control subjects ($0.04\% \pm 0.01\%$ versus $0.34\% \pm 0.1\%, P < .001$, Figure 1A,C). The frequency of CD4$^{+}$CD25$^{+}$ T cells (Figure 1A, gate a and b) from patients was also significantly decreased compared with the controls ($0.80\% \pm 0.32\%$ versus $2.48\% \pm 0.33\%, P < .001$, data not shown). Absolute Treg numbers from patients ($n = 20$) were significantly decreased (median, 0.24 cells/µL; range, 0.05-2.3 cells/µL) compared with the healthy donors’ absolute lymphocyte counts (available from 5 healthy control subjects; median, 5.9 cells/µL; range, 1.5-11 cells/µL).

In 13 cases, Treg and FOXP3 expression was reexamined 3 to 6 months after the first sampling; 6 of 7 patients who showed hematologic response to immunosuppressive treatment had slightly higher Treg numbers and FOXP3 expression compared with numbers obtained before treatment (data not shown). Three further patients in complete remission after immunosuppressive treatment had increased CD4$^{+}$CD25hi$^{-}$ T cells compared with treatment-naive patients (data not shown).

Decreased FOXP3 and NFA1 protein in CD4$^{+}$CD25$^{+}$ T cells from patients with acquired aplastic anemia

All patients examined ($n = 8$) had significantly decreased FOXP3 protein levels (FOXP3/actin OD: $0.31 \pm 0.1$ versus $1.03 \pm 0.1, P = .001$, Figure 1D,G and Figure S2) and FOXP3 mRNA/actin copies compared with healthy control subjects ($P = .03$). Horizontal lines represent mean values. (G-I) The densitometric intensities of immunoblot results from all the subjects studied for FOXP3, NFA1, and TLR2 expression are collectively presented. Horizontal lines represent mean values. Results are plus or minus standard error of the mean ($\pm$ SEM).
these complexes affect repressive effects on cytokine gene expression and their activating effects on Treg marker genes, CTLA4 and CD25. Based on the relationship between NFAT1 expression and FOXP3 established in vitro and in animal models,6,8 we examined this regulator’s expression in aplastic anemia; patients’ CD4+CD25+ T cells contained significantly decreased or absent NFAT1 protein levels compared with control subjects (NFAT1/actin OD: 0.29 ± 0.09 versus 1.1 ± 0.02, P < .001, Figure 1E,H, and Figure S2). The NFAT1 levels in CD4+CD25+ T cells were comparable between patients and control subjects (Figure 2C). There were no differences between patients and controls’ TLR2 protein levels21 (TLR2/actin OD: 1.06 ± 0.04 versus 1.05 ± 0.03, P = .9, Figure 1E,J).

These data were confirmed by confocal microscopy: CD4+CD25+ T cells from control subjects (n = 4) showed the expected pattern of NFAT1 and FOXP3 expression and localization. In patients, the results were consistent with the immunoblot and quantitative polymerase chain reaction experiment data; all patients examined (n = 5) had significantly decreased or absent FOXP3 and NFAT1 expression (Figure 2A).

To examine whether decreased FOXP3 correlated with NFAT1 expression, CD4+CD25+ T cells from patients and healthy control subjects were transiently transfected with an expression plasmid encoding for the wild-type NFAT1 gene. CD4+CD25+ T cells from patients previously examined and shown not to express any FOXP3 after transfection with the NFAT1 plasmid demonstrated increased levels of FOXP3 (Figure 2B). No difference in FOXP3 expression was observed in CD4+CD25+ T cells from healthy control subjects after transfection. CD4+CD25+ T cells from patients and control subjects also showed aberrant expression of FOXP3 after transfection compared with cells that were transfected with an empty GFP-plasmid as control (Figure S3). The transfected and the purified CD4+CD25+ and CD4+CD25− T cells were stimulated with anti-CD3 mAb or unperturbed and analyzed for interleukin-2 production. CD4+CD25+ T cells produced 4.5-fold more interleukin-2 after stimulation compared with the untreated population, whereas CD4+CD25− and the transfected CD4+CD25− T cells did not produce interleukin-2 after stimulation (data not shown). In cultures with CD25− T cells, the transfected cells could not suppress interleukin-2 production from CD4+CD25− T cells (data not shown). From these results, we inferred that FOXP3+CD25− cells generated after transient transfection were of an “anergic” phenotype as described for naturally occurring CD4+CD25+FOXP3+ T cells, but they were not suppressive.22 To confirm that decreased NFAT1 levels related to FOXP3 levels, we used CD4+CD25+ T cells from healthy control subjects in NFAT1 knockdown experiments. The NFAT1-knockdown CD4+CD25+ T cells showed decreased FOXP3 expression compared with the cells that were transfected with a control siRNA (Figure 2D), recapitulating a possible mechanism in patients’ cells by dysregulation of this critical regulator.

In summary, CD4+CD25+FOXP3+ regulatory T cells are decreased in most patients with aplastic anemia, possibly as a result of altered transcriptional regulation; decreased NFAT1 could explain low FOXP3 expression and Treg frequency. A role for NFAT1 might also be explored in other immune-mediated diseases in which Tregs have been implicated. Of course, alterations in other transcription factors that could influence FOXP3 expression are not excluded by our experiments and might also contribute to the decreased Treg numbers. A Treg defect may help explain the increased autoreactive T cells and the development of the aplastic anemia phenotype.

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Authorship

Contribution: E.E.S. designed and performed the research, analyzed data, and wrote the paper; K.R. and S.M. performed research,
analyzed data, and revised the paper; D.M. collected and analyzed data and revised the paper; K.K. provided technical support in flow cytometry experiments; V.V. and S.K. provided technical support; A.J.B. analyzed data and revised the paper; and N.S.Y. designed the research and wrote the paper.

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Deficient CD4+ CD25+ FOXP3+ T regulatory cells in acquired aplastic anemia

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