WASP regulates suppressor activity of human and murine $CD4^+CD25^+FOXP3^+$ natural regulatory T cells

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A large proportion of Wiskott-Aldrich syndrome (WAS) patients develop autoimmunity and allergy. $CD4^+CD25^+FOXP3^+$ natural regulatory T (nTreg) cells play a key role in peripheral tolerance to prevent immune responses to self-antigens and allergens. Therefore, we investigated the effect of WAS protein (WASP) deficiency on the distribution and suppressor function of nTreg cells. In *WAS*$^{-/-}$ mice, the steady-state distribution and phenotype of nTreg cells in the thymus and spleen were normal. However, *WAS*$^{-/-}$ nTreg cells engrafted poorly in immunized mice, indicating perturbed homeostasis. Moreover, *WAS*$^{-/-}$ nTreg cells failed to proliferate and to produce transforming growth factor β upon T cell receptor (TCR)/CD28 triggering. WASP-dependent F-actin polarization to the site of TCR triggering might not be involved in *WAS*$^{-/-}$ nTreg cell defects because this process was also inefficient in wild-type (WT) nTreg cells. Compared with WT nTreg cells, *WAS*$^{-/-}$ nTreg cells showed reduced in vitro suppressor activity on both WT and *WAS*$^{-/-}$ effector T cells. Similarly, peripheral nTreg cells were present at normal levels in WAS patients but failed to suppress proliferation of autologous and allogeneic $CD4^+$ effector T cells in vitro. Thus, WASP appears to play an important role in the activation and suppressor function of nTreg cells, and a dysfunction or incorrect localization of nTreg cells may contribute to the development of autoimmunity in WAS patients.

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Abbreviations used: HD, healthy donor; MFI, mean fluorescence intensity; nTreg, $CD4^+CD25^+FOXP3^+$ natural regulatory T; WAS, Wiskott-Aldrich syndrome; WASP, WAS protein.

Regulatory T cells play a key role in suppressing immune responses and in maintaining immunological homeostasis. Regulatory T cells have been shown to prevent autoimmune diseases, to down-modulate immune response to allergens, pathogens, and cancer cells, and to mediate peripheral transplantation tolerance (1–7). The best characterized subset of regulatory T cells are the $CD4^+CD25^+FOXP3^+$ natural regulatory T (nTreg) cells, whose differentiation, maintenance, and function are controlled by TCR engagement (8, 9), CD28 engagement (10, 11), FOXP3 (12, 13), and IL-2 (14, 15). nTreg cells are generated in the thymus upon TCR–peptide–MHC interaction (9) as a cell population with a broad repertoire of high-affinity TCRs, recognizing self-antigens (9, 16), tumor-associated antigens (17), allogeneic antigens (18), and possibly pathogen-derived antigens (19). Recent findings indicate that nTreg cells derive from high-affinity autoreactive T cells, which upon contact with a subset of activated DCs within the Hassal's corpuscles in the thymic medulla (20) acquire regulatory function. nTreg cells are readily present in the thymus, blood, and secondary lymphoid organs of healthy individuals or mice and represent 5–10% of the total $CD4^+$ T cells (2). The mechanisms by which nTreg cells are activated

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and mediate suppression of effector CD4+ and CD8+ T cells are still under investigation. Activation of nTreg cells is triggered by engagement of TCR and requires IL-2 (8, 21). In vitro suppressor activity of nTreg cells is mediated predominantly by cell–cell contact mechanisms. It remains to be clarified which are the molecules responsible for this process. A role for membrane-bound TGF-β (22) and CTLA-4 (23) in mediating nTreg cell suppressor function was hypothesized, but it appears to be limited to specific in vitro settings and was not confirmed by follow-up studies (24, 25). A possible additional mechanism of suppression could be via the release of perforin and granzyme by nTreg cells, resulting in apoptosis of effector T cells (26, 27). In addition, secretion of TGF-β (28, 29) and IL-10 (30) can contribute to the suppressor activity of nTreg cells in vivo. Quantitative and qualitative defects in regulatory T cells may result in pathology. Indeed, there is evidence that skewing of antigen-specific T cells toward a regulatory instead of a Th1 or Th2 phenotype is key in the maintenance of homeostasis and in the prevention of autoimmunity (31) and allergy (32).

Wiskott-Aldrich syndrome (WAS) is a primary human X-linked immunodeficiency caused by mutations in the WAS protein (WASP) (33) and characterized by recurrent infections and thrombocytopenia. A high proportion of WAS patients develop eczema and have high IgE levels (34), suggesting a Th2 unbalance. This is in line with the recent finding that WAS T cell lines have a selective defect in Th1 cytokine production (35) and that $WAS^{-/-}$ mice develop a skewed Th2 phenotype (Snapper, S.B., personal communication). Moreover, WAS patients lacking WASP expression have an increased risk of developing autoimmune diseases, including hemolytic anemia, cutaneous vasculitis, IgA nephropathy, inflammatory bowel disease, and arthritis (34, 36). WASP is a tightly regulated hematopoietic-specific protein, which controls actin nucleation (37), immunological synapse assembly (38), and migration to secondary lymphoid organs (39). In addition, WASP regulates apoptosis (40), proliferation, and IL-2 production in effector T cells (41–44). Although these defects contribute to the immunodeficiency, it cannot be excluded that defects in regulatory T cell populations and function are associated with the development of autoimmunity and with the unbalanced Th2 response in WAS patients. To determine whether the absence of WASP is associated with nTreg cell dysfunction, we characterized nTreg cells isolated from $WAS^{-/-}$ mice and WAS patients. In $WAS^{-/-}$ mice, the differentiation of nTreg cells and their steady-state distribution to secondary lymphoid organs were not significantly impaired. However, nTreg cell activation, in vitro suppressive activity, and in vivo engraftment were strongly reduced. Similarly, in WAS patients the frequency of peripheral nTreg cells was normal, but they displayed poor suppressor activity toward normal and WASP-deficient effector T cells in vitro. These data demonstrate that WASP is a major regulator of nTreg cell activation and function, suggesting that the autoimmune and atopic pathological manifestations in WAS may result from this impairment.

RESULTS

WASP and F-actin fail to polarize in murine nTreg cells after TCR triggering

To investigate whether WASP plays a role in the function of nTreg cells, we first analyzed WASP expression and localization in mouse WT nTreg cells. nTreg cells from the spleens of C57BL/6 mice express WASP at levels comparable to that of CD4+CD25− T cells, CD8+ T cells (Fig. 1 a), B cells, NK cells, and granulocytes (not depicted). We previously showed that WASP promotes actin polarization at the immunological synapse of effector T cells (38). Accordingly, after activation of WT CD4+CD25− effector T cells with WT APCs in the presence of anti-CD3 mAbs, F-actin and WASP polarized to the APC contact site in ~50% of the conjugates (Fig. 1, b and c). In contrast, <10% of CD4+CD25+ WT nTreg

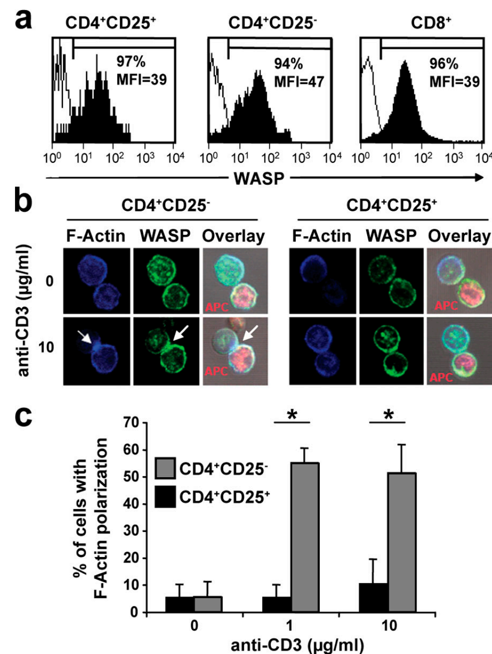

Figure 1. WASP expression in murine nTreg cells. (a) WASP expression in CD4+CD25+, CD4+CD25−, and CD8+ T cells. Splenocytes from WT mice were stained with anti-WASP mAbs (filled histogram) or isotype matched control antibodies (empty histogram) and gated on the indicated T cell subset. Numbers indicate percentages and MFI of WASP+ cells. (b) F-actin and WASP localization in murine splenic WT CD4+CD25− and CD4+CD25+ T cells upon stimulation. CD4+CD25− effector T cells (left) and CD4+CD25+ nTreg cells (right) were stimulated with WT APCs (stained in orange) in the absence or presence of 10 μg/ml anti-CD3 mAbs. Images showing F-actin staining (left column), WASP staining (middle column), and their bright field overlay (right column) are shown. These images are representative of at least 100 T cell–APC conjugates that were evaluated for each experimental condition. White arrows indicate F-actin and WASP polarization at the T cell–APC interface. (c) Percentage of WT CD4+CD25+ nTreg cells and WT CD4+CD25− effector T cells showing F-actin polarization at the T cell–APC interface in the presence of APCs and in the absence or presence of the indicated amount of anti-CD3 mAbs. Histogram bars represent the average percentage (± SD) of F-actin polarization in T cells from three independent experiments. *, $P < 0.05$, Student's *t* test.

cells polarized F-actin and WASP at the contact of APCs. In most nTreg cells, a ring-like distribution of F-actin and WASP beneath the plasma membrane was observed (Fig. 1 b). The reduced polarization of F-actin and WASP in nTreg cells was not due to their defective ability to form conjugates with APCs because similar proportions of nTreg cells and effector T cells were in contact with APCs (not depicted). These data indicate that murine nTreg cells express levels of WASP similar to those of $CD4^+CD25^-$ effector T cells, but they do not efficiently polarize F-actin and WASP to the APC contact site upon TCR triggering, suggesting that WASP-dependent actin polarization at the immunological synapse does not occur in nTreg cells under these in vitro conditions.

Normal differentiation and steady-state tissue distribution of nTreg cells in *WAS*$^{-/-}$ mice

To determine whether WASP is required for nTreg cell development, Foxp3 expression was analyzed in double negative, double positive, $CD4^+$ single positive, and $CD8^+$ single positive thymocytes of *WAS*$^{-/-}$ mice. Comparable proportions of Foxp3-expressing $CD4^+$ single positive and double positive thymocytes were observed in WT and *WAS*$^{-/-}$ mice. The vast majority of $CD24^{lo}Foxp3^+$ mature thymocytes were contained in the $CD4^+$ single positive T cells in both WT and *WAS*$^{-/-}$ mice (Fig. 2 a). In addition, the overall thymic cellularity was similar in WT and *WAS*$^{-/-}$ mice (not depicted). Moreover, the relative percentages and absolute counts of $CD4^+CD8^-CD25^+$ T cells, as well as the mean fluorescence intensity (MFI) of CD25, were also comparable (Fig. 2, b and c). A raise in the absolute count of $CD4^+CD25^-Foxp3^+$ and $CD4^+CD25^+Foxp3^+$ T cells was found in the thymi of *WAS*$^{-/-}$ mice as compared with WT controls (Fig. 2, d and e). Collectively, these data indicate that thymic differentiation of nTreg cells can efficiently occur in *WAS*$^{-/-}$ mice.

We next investigated if WASP deficiency may influence the steady-state distribution of nTreg cells into the spleen. The overall splenocyte count was comparable in WT and *WAS*$^{-/-}$ mice (not depicted). Normal percentages and numbers

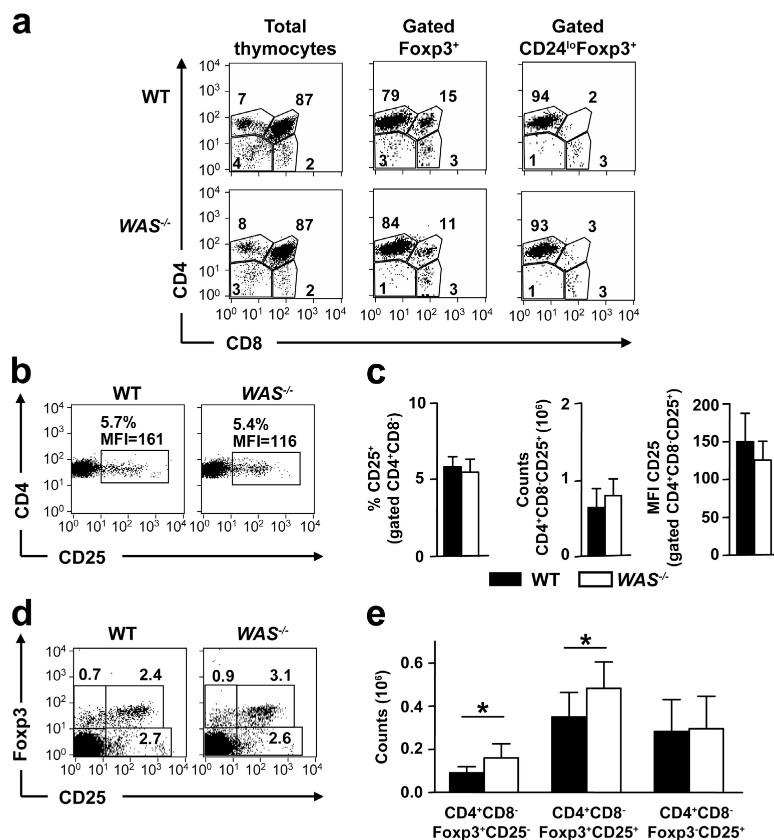

Figure 2. Thymic generation of nTreg cells in *WAS*$^{-/-}$ mice. (a) Thymic development of nTreg cells in WT and *WAS*$^{-/-}$ mice. CD4/CD8 flow cytometric plots were gated on live thymocytes, total $Foxp3^+$ cells, and mature $CD24^{lo}Foxp3^+$ cells. Numbers indicate the percentage of cells in the respective region. Data are representative of 12 mice per group analyzed in three independent experiments. (b) Immunophenotype of $CD4^+$ single positive thymocytes from representative WT and *WAS*$^{-/-}$ mice. Numbers indicate the percentages and MFI of $CD25^+$ cells. (c) Percentage, absolute count, and MFI of $CD25^+$ cells among $CD4^+$ single positive thymocytes. Mean ± SD of 13 mice per group are shown. (d) Foxp3 and CD25 expression in $CD4^+$ single positive thymocytes. Numbers indicate the percentage of cells in the respective region. (e) Absolute count of the indicated thymocyte populations. Mean ± SD of 13 mice per group is shown. *, $P < 0.05$, Student's *t* test.

of CD4$^+$CD25$^+$ T cells were found in the spleens of *WAS*$^{-/-}$ mice, although the expression of CD25 was reduced as compared with WT mice (Fig. 3, a and b). As in the thymus, the absolute count of CD4$^+$CD25$^-$Foxp3$^+$ T cells increased in the spleens of *WAS*$^{-/-}$ mice. Conversely, the numbers of splenic CD4$^+$CD25$^+$Foxp3$^+$ nTreg cells were comparable to those of normal mice, whereas an increased number of splenic CD4$^+$CD25$^+$Foxp3$^-$ T cells was observed (Fig. 3, c and d). Expression of the nTreg cell markers CTLA-4 and GITR on CD4$^+$CD25$^+$ splenic T cells was in the normal range, indicating that *WAS*$^{-/-}$ nTreg cells have a normal phenotype (Fig. 3 e). *WAS*$^{-/-}$ CD4$^+$CD25$^+$ T cells also expressed normal levels of the activation markers CD69, CD62L, and CD45RB (Fig. 3 e). These data indicate that splenic nTreg cells are present in *WAS*$^{-/-}$ mice in a similar amount to those present in WT mice, albeit CD25 expression is reduced.

Defective suppressor activity of murine *WAS*$^{-/-}$ nTreg cells

To investigate whether *WAS*$^{-/-}$ nTreg cells are functional, proliferation of effector T cells in the presence of WT or *WAS*$^{-/-}$ nTreg cells was tested. As expected, WT nTreg cells suppressed proliferation of WT CD90$^+$CD25$^-$ effector T cells activated with anti-CD3 mAbs in a cell dose–dependent fashion (Fig. 4 a, filled bars). In contrast, *WAS*$^{-/-}$ nTreg cells had a reduced capacity to suppress WT effector T cell proliferation at every ratio of effector T cells to nTreg cells tested (Fig. 4 a, dotted vs. filled bars). These data indicate that nTreg cells require WASP to suppress WT effector T cells in vitro. The susceptibility of *WAS*$^{-/-}$ effector T cells, compared with WT effector T cells, to be suppressed by WT nTreg cells (Fig. 4 a, dashed vs. filled bars) or by *WAS*$^{-/-}$ nTreg cells (Fig. 4 a, empty vs. filled bars) was also tested. *WAS*$^{-/-}$ effector T cells proliferated at a lower extent than WT effector cells (median proliferation was 6,360 and 26,380 cpm, respectively; $P < 0.05$ as determined by Mann-Whitney test). Suppression of proliferation of *WAS*$^{-/-}$ effector T cells by WT nTreg cells was comparable to the suppression observed with WT effector T cells when an effector T cell/nTreg cell ratio of 1:0.16 was used. At lower effector T cell/nTreg cell ratios, suppression of *WAS*$^{-/-}$ effector T cells by WT nTreg cells was significantly lower compared with that of WT effector T cells (Fig. 4 a, dashed vs. filled bars). Similar results were obtained with *WAS*$^{-/-}$ nTreg cells (Fig. 4 a, empty vs. filled bars). These data indicate that proliferation of *WAS*$^{-/-}$ effector T cells was not suppressed by WT or *WAS*$^{-/-}$ nTreg cells in a cell dose–dependent manner. This may be due to intrinsic defects of

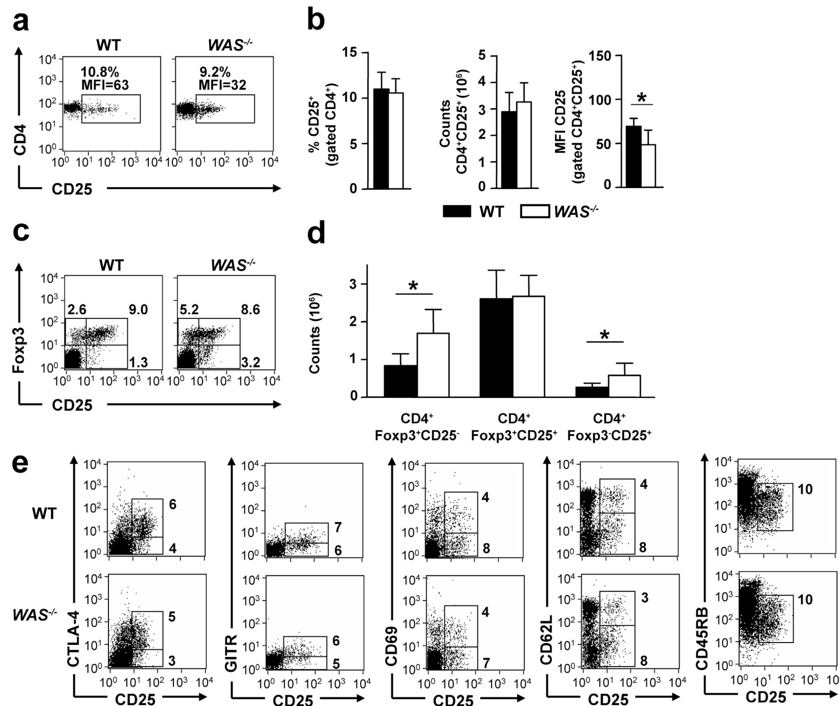

Figure 3. Cell count and immunophenotype of nTreg cells in the spleens of *WAS*$^{-/-}$ mice. (a) Immunophenotype of CD4$^+$ splenocytes from representative WT and *WAS*$^{-/-}$ mice. Numbers indicate the percentages and MFI of CD25$^+$ cells. (b) Percentage, absolute count, and MFI of CD25$^+$ cells among CD4$^+$ splenocytes. Mean ± SD of 13 mice per group is shown. *, $P < 0.05$, Student's *t* test. (c) Foxp3 and CD25 expression in CD4$^+$ splenocytes. Numbers indicate the percentage of cells in the respective region. (d) Absolute count of the indicated splenocyte populations. Mean ± SD of 13 mice per group is shown. *, $P < 0.05$, Student's *t* test. (e) Immunophenotype of nTreg cells from WT and *WAS*$^{-/-}$ mice. Expression of CD25 is shown together with the expression of CTLA-4, GITR, CD69, CD62L, and CD45RB. Negative control staining resulted in signal below the value of 10. Numbers indicate the percentages of cells in the respective regions. Data are representative of six to eight mice per group analyzed in two independent experiments.

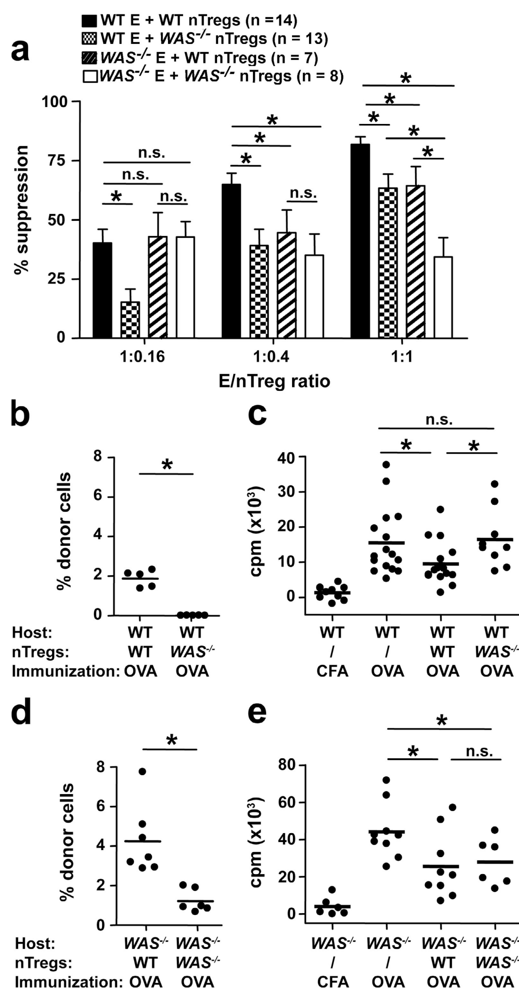

Figure 4. Suppressor activity of *WAS*$^{-/-}$ nTreg cells in vitro and in vivo. (a) In vitro suppression of WT or *WAS*$^{-/-}$ CD90$^+$CD25$^-$ effector T cells (abbreviated as WT E and *WAS*$^{-/-}$ E, respectively). Effector T cells were mixed with purified CD4$^+$CD25$^+$ nTreg cells isolated from WT or *WAS*$^{-/-}$ mice at the indicated ratios and stimulated with 10 μg/ml of plate-bound anti-CD3 mAbs. Median proliferation of effector T cells: WT = 26,380 cpm and *WAS*$^{-/-}$ = 6,360 cpm. Mean percentage of suppression ± SEM of the indicated number of independent experiments is shown. *, P < 0.05, Student's *t* test. (b and d) Tracking of adoptively transferred nTreg cells. Purified nTreg cells from WT or *WAS*$^{-/-}$ mice were adoptively transferred into WT (b) or *WAS*$^{-/-}$ (d) recipients, which were immunized with OVA the day after. Percentage of CD45.2-expressing donor CD4$^+$Foxp3$^+$ cells in the draining LN was determined after 7–10 d. Bars represent the average value for each group. *, P < 0.05, Student's *t* test. (c and e) In vitro recall OVA-specific proliferation by total cells recovered from the draining LNs of WT (c) or *WAS*$^{-/-}$ (e) mice treated as in b and d. When mice were killed, total LN cells were restimulated with OVA for 72 h. Plotted values are cpm for each individual mouse with background subtraction. Bars represent the average value for each group. *, P < 0.05, Student's *t* test.

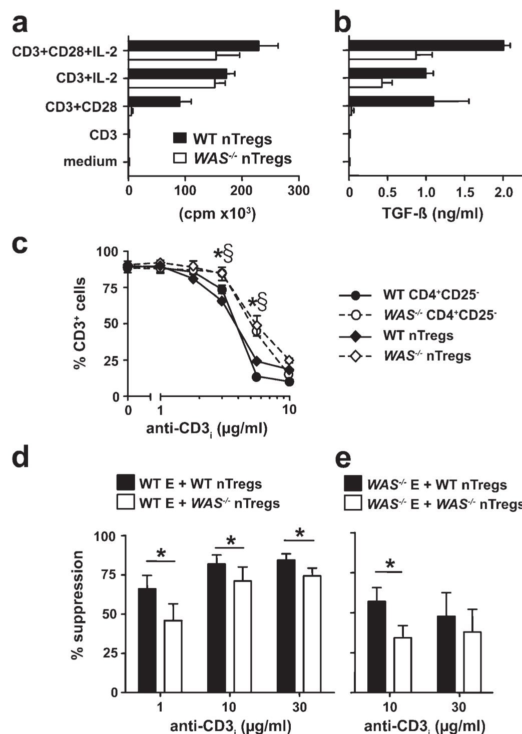

Figure 5. TCR-mediated activation of *WAS*$^{-/-}$ nTreg cells. (a) Proliferation of WT and *WAS*$^{-/-}$ nTreg cells stimulated with 10 μg/ml of plate-bound anti-CD3 mAbs, 100 U/ml IL-2, and/or 1 μg/ml anti-CD28 mAbs as indicated. The average cpm ± SEM of three independent experiments is shown. (b) TGF-β production by WT and *WAS*$^{-/-}$ nTreg cells activated as in a. The average concentration ± SEM of three independent experiments is shown. (c) TCR down-regulation in nTreg cells and CD4$^+$CD25$^-$ effector T cells after stimulation with anti-CD3 mAbs. Plotted values represent the average percentage of CD3$^+$ cells of three mice per group. Error bars represent SD. For nTreg cells, *, P < 0.05. For effector CD4$^+$CD25$^-$ T cells, §, P < 0.05, Student's *t* test. One representative experiment out of three is shown. (d and e) Suppressor activity of WT and *WAS*$^{-/-}$ CD4$^+$CD25$^+$ nTreg cells on WT (d) or *WAS*$^{-/-}$ effector T cells (e) stimulated with different concentrations of anti-CD3 mAbs. Effector T cells (E) were cocultured with nTreg cells at 1:1 ratio and stimulated with the indicated doses of anti-CD3 mAbs. For WT effectors, median proliferation was 46,400 cpm at 1 μg/ml anti-CD3, 35,260 cpm at 10 μg/ml anti-CD3, and 29,000 cpm at 30 μg/ml anti-CD3. For *WAS*$^{-/-}$ effectors, median proliferation was 6,240 cpm at 10 μg/ml anti-CD3 and 7,630 cpm at 30 μg/ml anti-CD3. For each dose of anti-CD3 mAbs, mean percentage of suppression ± SEM is shown. For d, *n* > 10; for e, *n* > 6. *, P < 0.05, paired Student's *t* test.

WAS$^{-/-}$ effector T cells in providing factors required for activation of nTreg cells.

The in vivo ability of adoptively transferred *WAS*$^{-/-}$ and WT nTreg cells to engraft and modulate an antigen-specific immune response upon OVA immunization in WT or *WAS*$^{-/-}$ mice was investigated. 7–10 d after OVA immunization of WT recipients, donor WT nTreg cells (transferred 1 d before immunization) represented ~2% of the CD4$^+$Foxp3$^+$ (Fig. 4 b) or CD4$^+$CD25$^+$ cells (not depicted) in the draining LNs. In contrast, donor *WAS*$^{-/-}$ nTreg cells could not be recovered in draining LNs (Fig. 4 b). The same results were obtained when donor nTreg cells were counted in nondraining LNs, spleen, blood, liver, and lungs regardless of CD25 and Foxp3 expression (not depicted). Accordingly, the in vitro proliferative recall responses to OVA were significantly

Table I. WAS gene mutations and clinical status of WAS patients

Patient	Age (yr)[a]	Mutation type	gDNA mutation	Protein change	Score[b]	Thrombo-cytopenia	T cell lymphopenia	Eczema	Infections	Autoimmunity
WAS1	24	Splice Intron 9	IVS9 +2 del tgag	ND	4	Yes	No	Mild	Severe HSV, pneumonia	No
WAS4	23	Deletion Exon 6	570del t	fs stop aa260	5	Yes[c]	Yes	Mild	Various and recurrent infections	Vasculitis, arthritis, IgA nephropathy
WAS13	13.8	Missense Exon 1	150t>c	L39P	2	Yes[c]	Yes	Mild	No	No
WAS14	1.8	Missense Exon 4	431g>a	E133K	5	Yes	Yes	Mild	No	Colitis, vasculitis[d]
WAS17	2.8	Splice Intron 8	IVS8 +1 del gtga	fs stop aa246	4	Yes	No	Severe	Recurrent otitis, pneumonia	No
WAS21	18	Missense Exon 7	741c>g	A236G	2	Yes[c]	Yes	No	Recurrent otitis	No
WAS22	19	Missense Exon 2	201c>t	A56V	1	Yes	No	No	No	No

fs, frameshift; del, deletion; IVS, intervening sequence (intron); ND, not determined.
[a]Age refers to the time of blood sampling.
[b]Disease score is given according to the classification reported previously (reference 34).
[c]Splenectomized.
[d]Patient undergoing therapy with prednisone.

suppressed after adoptive transfer of WT nTreg cells, but not of $WAS^{-/-}$ nTreg cells (Fig. 4 c). Significantly lower percentages of $CD4^+Foxp3^+$ (Fig. 4 d) or $CD4^+CD25^+$ (not depicted) $WAS^{-/-}$ nTreg cells as compared with WT nTreg cells were also recovered in $WAS^{-/-}$ recipients (1 and 4%, respectively). However, both WT and $WAS^{-/-}$ nTreg cells modulated the OVA-specific immune response elicited in $WAS^{-/-}$ mice, as demonstrated by the in vitro recall response to OVA (Fig. 4 e). It should be noted that the overall proliferation of $WAS^{-/-}$ T cells to OVA was higher than that of WT T cells, even when the $WAS^{-/-}$ nTreg cells were transferred in vivo. Collectively, our results indicate that WASP-deficient nTreg cells fail to engraft in WT and displayed a reduced engraftment in $WAS^{-/-}$ hosts, suggesting that this defect may contribute to their dysfunction in vivo.

Impaired TCR/CD28 activation of $WAS^{-/-}$ nTreg cells

Activation through the TCR is required for the suppressive function of nTreg cells (8). Therefore, it is possible that defective suppressor activity of $WAS^{-/-}$ nTreg cells is due to a defect in their activation. To address this question, we measured the proliferative response and the cytokine secretion profile of $WAS^{-/-}$ nTreg cells after TCR stimulation. As expected, neither WT nor $WAS^{-/-}$ nTreg cells proliferated (Fig. 5 a) or produced TGF-β (Fig. 5 b) in response to anti-CD3 mAbs alone. Furthermore, in contrast to WT nTreg cells, $WAS^{-/-}$ nTreg cells failed to proliferate and secrete TGF-β after activation with anti-CD3 plus anti-CD28 mAbs (Fig. 5, a and b). The addition of exogenous IL-2 to anti-CD3 or anti-CD3 plus anti-CD28 mAbs stimulation induced comparable proliferation of WT and $WAS^{-/-}$ nTreg cells (Fig. 5 a) and promoted TGF-β production, although at reduced levels in $WAS^{-/-}$ nTreg cells (Fig. 5 b). Neither WT nor $WAS^{-/-}$ nTreg cells produced IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α after stimulation through the TCR/CD28 (not depicted). These data show that $WAS^{-/-}$ nTreg cells have a major activation defect downstream of the TCR and the CD28 receptor, and that proliferation, but not TGF-β production, is fully restored by the addition of exogenous IL-2. To determine whether $WAS^{-/-}$ nTreg cells have a higher threshold for TCR-mediated activation as compared with WT nTreg cells, we measured TCR down-regulation after stimulation with increasing doses of anti-CD3 mAbs. Both WT nTreg cells and effector T cells down-regulated CD3 in a comparable dose-dependent manner (Fig. 5 c). CD3 was also down-regulated in $WAS^{-/-}$ nTreg cells and effector T cells, but higher doses of anti-CD3 mAbs were required to obtain levels of down-regulation similar to those observed in WT cells (Fig. 5 c). We next investigated whether the impaired regulatory function of $WAS^{-/-}$ nTreg cells could depend on the strength of TCR stimulation. WT effector T cells were suppressed by WT nTreg cells in an anti-CD3 mAb dose-dependent manner (Fig. 5 d). On the other hand, $WAS^{-/-}$ nTreg cells had a reduced suppressive ability compared with WT nTreg cells at the dose of 1 μg/ml, but the suppressive capacity was enhanced when the anti-CD3 mAb dose was increased (Fig. 5 d). However, the suppressive activity of $WAS^{-/-}$ nTreg cells could never reach normal levels (Fig. 5 d). Subsequently, increasing doses of anti-CD3 mAbs were used to activate $WAS^{-/-}$ effector T cells to determine whether higher levels of activation render these cells more prone to nTreg cell–mediated suppression. $WAS^{-/-}$ effector T cells failed to proliferate after stimulation with 1 μg/ml anti-CD3 mAbs

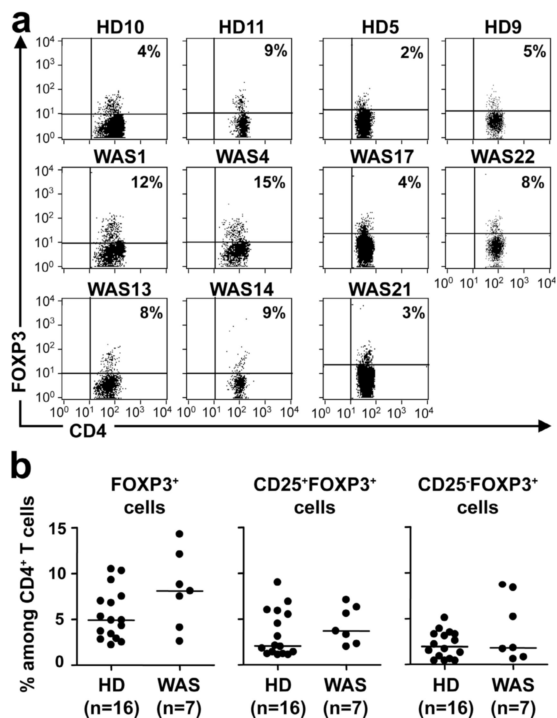

Figure 6. Immunophenotype of nTreg cells from human peripheral blood. (a) $CD4^+FOXP3^+$ cells in the peripheral blood of WAS patients. PBMCs from four representative HDs and seven WAS patients (WAS) were stained with anti-CD4 and anti-FOXP3 mAbs. Results shown are gated on the $CD4^+$ T cells. Numbers indicate the percentages of $FOXP3^+$ cells among $CD4^+$ T cells. (b) Percentage of $FOXP3^+$ cells (left), $CD25^+FOXP3^+$ cells (middle), and $CD25^-$ $FOXP3^+$ cells (right) among $CD4^+$ T cells of HDs (n = 16) and WAS patients (n = 7). Bars represent the median value for each group.

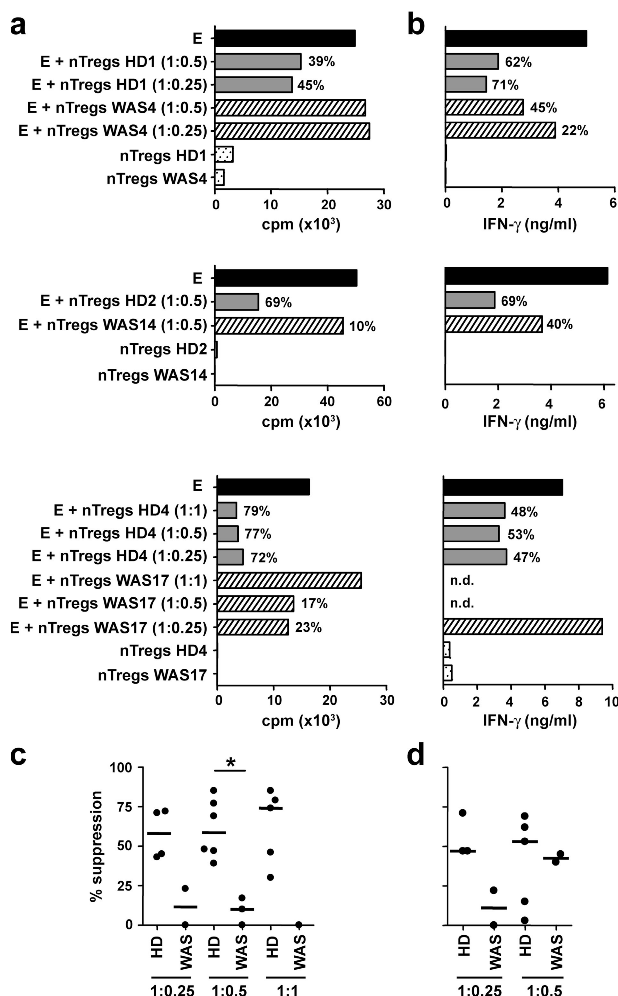

Figure 7. Allogeneic suppression by human nTreg cells. (a and b) Ability of nTreg cells freshly isolated from WAS patients and HDs to suppress allogeneic $CD4^+CD25^-$ effector T cells from HDs. Effector T cells were cultured in the presence of nTreg cells at the indicated ratio and stimulated with CD3-depleted irradiated allogeneic APCs and anti-CD3 mAbs for 72 h (HD1 and WAS4), anti-CD3 mAbs plus anti-CD28 mAb–coated beads for 72 h (HD2 and WAS14), or anti-CD3 mAbs plus anti-CD28 mAb–coated beads for 120 h (HD4 and WAS17). Proliferation was measured by [^{3}H]thymidine incorporation (a), and IFN-γ secretion was measured by cytometric bead array (b). Filled bars, activated effector T cells (abbreviated as E); gray bars, activated effector T cells in the presence of nTreg cells isolated from HDs; dashed bars, activated effector T cells in the presence of nTreg cells isolated from WAS patients; dotted bars, activated nTreg cells alone. Percentages of suppression are indicated. (c and d) Cumulative graph of allogeneic suppression of proliferation (c) and IFN-γ production (d) at the indicated effector T cell/nTreg cell ratio. Dots represent percentage of suppression of each HD or WAS patient, and bars depict the respective median value. *, $P < 0.05$, Mann-Whitney test.

(not depicted). At the dose of 10 μg/ml anti-CD3 mAbs, $WAS^{-/-}$ nTreg cells suppressed $WAS^{-/-}$ effector T cells less efficiently than WT nTreg cells (Fig. 5 e). An increase in the anti-CD3 mAbs dose (30 μg/ml) led to comparable levels of suppression of $WAS^{-/-}$ effector T cells by WT and $WAS^{-/-}$ nTreg cells (Fig. 5 e), although the suppressive activity was lower than that observed with WT effector T cells. Overall, these data indicate that the suppressive defect of $WAS^{-/-}$ nTreg cells can depend both on the strength of TCR stimulation and on the dysfunction of $WAS^{-/-}$ effector T cells, suggesting an important role of WASP in both effector and regulatory T cells.

Normal percentage of $CD4^+CD25^+FOXP3^+$ nTreg cells in the blood of WAS patients

To our knowledge, the role of WASP in the generation and function of human nTreg cells has never been studied. As observed in murine cells, levels of WASP expression in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the blood of healthy donors (HDs) were comparable (not depicted). To investigate the role of WASP in the generation of human nTreg cells, we analyzed $CD4^+CD25^+FOXP3^+$ T cells from the peripheral blood of seven WAS patients of different ages and different clinical scores (Table I). Percentages of $FOXP3^+$ cells among $CD4^+$ T cells of WAS patients were either higher or comparable to those observed in HDs (Fig. 6 a). Overall analysis revealed a slight increase in $FOXP3^+$ cells within

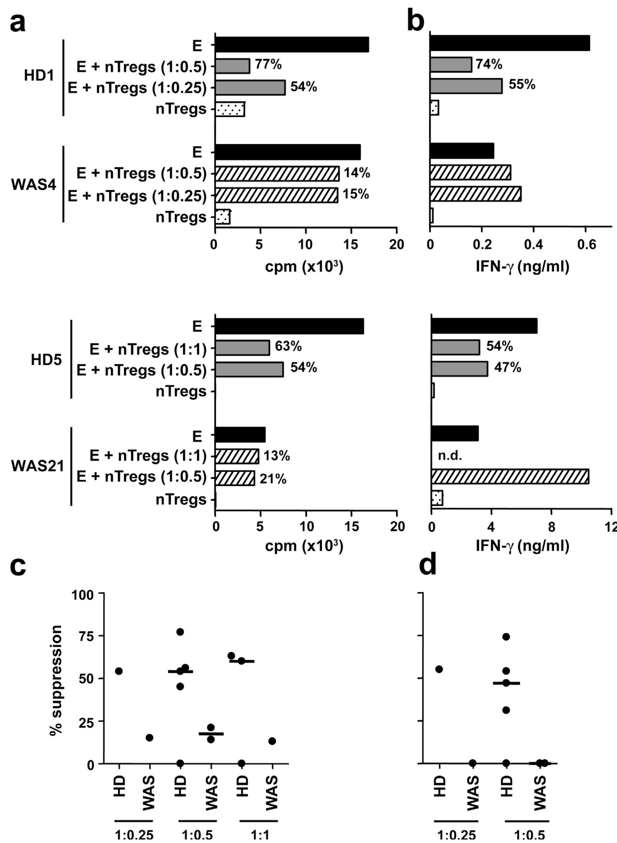

Figure 8. Autologous suppression by human nTreg cells. (a and b) Ability of nTreg cells freshly isolated from WAS patients and HDs to suppress autologous $CD4^+CD25^-$ effector T cells. Effector T cells were cultured in the presence of nTreg cells at the indicated ratio and stimulated with CD3-depleted irradiated allogeneic APCs and anti-CD3 mAbs for 72 h (HD1 and WAS4), or with anti-CD3 mAbs plus anti-CD28 mAb–coated beads for 120 h (HD5 and WAS21). Proliferation was measured by [^{3}H]thymidine incorporation (a), and IFN-γ secretion was measured by cytometric bead array (b). Filled bars, activated effector T cells (abbreviated as E); gray bars, activated effector T cells in the presence of nTreg cells isolated from HDs; dashed bars, activated effector T cells in the presence of nTreg cells isolated from WAS patients; dotted bars, activated nTreg cells alone. Percentages of suppression are indicated. (c and d) Cumulative graph of autologous suppression of proliferation (c) and IFN-γ production (d) at the indicated effector T cell/nTreg cell ratio. Dots represent percentage of suppression of each HD or WAS patient, and bars depict the respective median value.

patients' $CD4^+$ T lymphocytes, which is mainly due to an increase in $CD4^+CD25^+FOXP3^+$ T cells (Fig. 6 b). The MFI values of CD25 and FOXP3 in $CD4^+$ T cells from WAS patients were comparable to those measured in HDs (not depicted). These results indicate that WAS patients have a normal or increased proportion of circulating nTreg cells.

Defective suppressor activity of nTreg cells from WAS patients

Next, we investigated whether nTreg cells from WAS patients were able to suppress normal allogeneic effector T cells. nTreg cells isolated from three WAS patients failed to suppress proliferation of allogeneic effector T cells (Fig. 7 a), whereas suppression of IFN-γ production was reduced in two out of three WAS patients and completely defective in the third patient (Fig. 7 b). As expected, nTreg cells isolated from HDs had significant suppressor activity toward normal allogeneic $CD4^+CD25^-$ effector T cells, resulting in reduced proliferation (Fig. 7 a) and IFN-γ secretion (Fig. 7 b). Upon stimulation, nTreg cells from WAS patients and HDs were anergic and failed to proliferate (Fig. 7 a) and to produce IFN-γ (Fig. 7 b). Analysis of all experiments performed showed that nTreg cells from WAS patients were consistently impaired in their ability to suppress proliferation of allogeneic targets (Fig. 7 c), whereas a residual suppression of IFN-γ production could be detected (Fig. 7 d). We also tested the suppressor activity of nTreg cells from WAS patients toward autologous $CD4^+CD25^-$ effector T cells. Although control nTreg cells strongly suppressed the proliferation (Fig. 8 a) and IFN-γ secretion (Fig. 8 b) of autologous effector T cells, nTreg cells from two WAS patients tested had no significant suppressive effects on cell proliferation (Fig. 8 a) and IFN-γ secretion (Fig. 8 b) by autologous effector T cells. Overall, we conclude that nTreg cells from WAS patients were impaired in suppressing both proliferation (Fig. 8 c) and IFN-γ production (Fig. 8 d) of autologous targets.

Collectively, these data demonstrate that nTreg cells from WAS patients are strongly impaired in their suppressor activity toward both autologous and allogeneic targets.

DISCUSSION

In this study, we identify WASP as a key molecule for the optimal function of human and murine nTreg cells. WASP appears to be dispensable for thymic development and steady-state distribution of nTreg cells to the periphery. However, WASP is required for the TCR/CD28-triggered proliferation, the TGF-β production, and the suppressive function of nTreg cells. Given the key role played by these cells in maintaining peripheral tolerance, our data suggest that nTreg cell dysfunction might contribute to the high susceptibility of WAS patients to develop allergy and multiple autoimmune disorders.

Normal proportions of nTreg cells were found in the thymus of $WAS^{-/-}$ mice, and the induction of Foxp3 expression occurred at the expected stage of thymocyte maturation. In addition, no abnormalities in thymic architecture, including the ratio between cortical and medullary areas and the number of Hassal's corpuscles, were observed in $WAS^{-/-}$ mice (not depicted). The normal thymic differentiation of WASP-deficient nTreg cells suggests that mechanisms of positive and negative selection of T cells with high-affinity TCR are preserved, including the TCR-dependent conversion of autoreactive T cells into nTreg cells (5). This hypothesis is consistent with a minor role of WASP in thymic T cell differentiation, as suggested by previous studies in C57BL/6 $WAS^{-/-}$ mice (45) and in patients (46).

nTreg cells generated in the thymus undergo a robust peripheral expansion that is mainly dependent on paracrine delivery of IL-2 by activated T cells and possibly DCs (47). The IL-2–IL-2R axis appears to be dispensable during thymic development, but it is required for the survival of nTreg cells in the periphery (14, 15). Although WAS is characterized by defective IL-2 production by T cells (41–44), no reduction in the relative numbers of nTreg cells in the spleens of $WAS^{-/-}$ mice was observed. These findings suggest that low levels of IL-2 in $WAS^{-/-}$ mice are sufficient to provide the appropriate survival signals to peripheral nTreg cells. Although normal numbers of nTreg cells were found in the spleens of $WAS^{-/-}$ mice, adoptive transfer of $WAS^{-/-}$ nTreg cells into WT or $WAS^{-/-}$ hosts resulted in no or reduced, respectively, recovery of those cells in draining LNs after immunization. This defective engraftment of $WAS^{-/-}$ nTreg cells could be due to impaired migration, survival, and/or proliferation, and it may be even more evident in the competitive environment represented by WT hosts. In a distinct WASP-deficient murine strain, Maillard et al. (reference 48; p. 379 of this issue) found decreased numbers of nTreg cells in the spleen, suggesting that the degree of defects in nTreg cell homeostasis may depend on the genetic background of the $WAS^{-/-}$ mouse model.

We showed here that murine WASP-deficient nTreg cells were impaired in their regulatory function. Indeed, in vitro experiments showed that $WAS^{-/-}$ nTreg cells had a significantly reduced capacity to suppress WT effector T cell proliferation at every cell ratio tested. After strong TCR stimulation (30 μg/ml anti-CD3 mAbs), a residual suppressor activity of $WAS^{-/-}$ nTreg cells on WT effector T cells could be detected, suggesting that the in vitro dysfunction of $WAS^{-/-}$ nTreg cells is mainly due to an activation defect. Previous studies have shown that the suppressor activity of nTreg cells directly depends on their activation through the TCR (8). Indeed, we clearly demonstrated that $WAS^{-/-}$ nTreg cells have a profound defect in TCR/CD28-mediated activation. Upon stimulation with anti-CD3 and anti-CD28 mAbs, $WAS^{-/-}$ nTreg cell proliferation and TGF-β production were abolished. It remains to be determined whether the defect in TGF-β production contributes to the dysfunctional nTreg cell activity.

Given the master role of WASP in immunological synapse assembly, it is tempting to speculate that WASP contributes to the assembly of an activatory synapse in nTreg cells and that the lack of this protein prevents optimal TCR activation in nTreg cells. However, we showed that, in contrast to effector T cells, murine nTreg cells failed to polarize F-actin and concomitantly WASP to the site of contact with anti-CD3 mAb-loaded APCs or beads coated with anti-CD3 and anti-CD28 mAbs (not depicted). Therefore, a role of WASP cannot be demonstrated in this process. On the other hand, it cannot be excluded that nTreg cells assemble a structurally different immunological synapse, or that its assembly follows different kinetics. It is also possible that the activation and functional defects of $WAS^{-/-}$ nTreg cells are independent from immunological synapse formation and are due to defects in signaling downstream of the TCR. The observation that defective suppressive function in $WAS^{-/-}$ nTreg cells was present at a dose of TCR stimulation leading to normal TCR internalization is consistent with the hypothesis that additional defects in late TCR-mediated signaling may play a role in the activation of these cells (49).

The activation state of the effector T cells may also play a role in the suppressor activity of nTreg cells. We showed that WT nTreg cells could normally suppress the proliferation of $WAS^{-/-}$ effector T cells at the effector T cell/nTreg cell ratio of 1:0.16, but at lower ratios, the suppressor activity was reduced as compared with that observed in cultures with WT effector T cells. These data suggest a lack of activatory signals by $WAS^{-/-}$ effector T cells. $WAS^{-/-}$ nTreg cells' failure to suppress autologous $WAS^{-/-}$ effector T cells was even more profound than that of WT nTreg cells (ratio 1:1), supporting a greater dependence of the former on signals delivered by effector T cells that are required for their activation. Therefore, we propose that defective activation of $WAS^{-/-}$ effector T cells, which leads to impaired production of IL-2, might further amplify the intrinsic defect of $WAS^{-/-}$ nTreg cells. In line with this hypothesis, a defective cytokine production by effector T cells isolated from patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome was recently proposed to contribute to the dysfunction of nTreg cells in this disease (50). We found that $WAS^{-/-}$ nTreg cells were responsive to TCR/CD28 stimulation in the presence of exogenous IL-2. In a complementary study, administration of exogenous IL-2 to anti-CD3 mAb–stimulated $WAS^{-/-}$ nTreg cells could strongly enhance their suppressive ability in vitro (48). Collectively, these data suggest that in $WAS^{-/-}$ mice, conditions leading to non-optimal activation of effector T cells and defective IL-2 production might amplify the intrinsic activation defect of nTreg cells.

Recent data demonstrated that transfer of a non-antigen–specific WT nTreg cell population in normocytic mice was able to control antigen-specific responses (51). In our study, we compared the ability of adoptively transferred WT versus $WAS^{-/-}$ nTreg cells to modulate an in vivo immune response to OVA. $WAS^{-/-}$ nTreg cells transferred in WT recipients were not found in LNs draining the site of OVA immunization, and subsequently, modulation of the OVA-specific immune response was not observed. Redistribution of $WAS^{-/-}$ nTreg cells transferred in autologous $WAS^{-/-}$ recipients was reduced, and in this condition, down-modulation of the OVA-specific immune response was still observed. It should be noted that in the immunization protocol used here, the proliferation of $WAS^{-/-}$ effector T cells upon in vitro restimulation with OVA was significantly higher compared with that observed in WT mice. This could be related to a status of spontaneous immune activation in $WAS^{-/-}$ mice, as illustrated by the presence of increased numbers of $CD4^{+}CD25^{+}Foxp3^{-}$ splenocytes. These conditions of potent immune reaction might therefore be permissive for suppression

by $WAS^{-/-}$ nTreg cells, although the recall response of cells from $WAS^{-/-}$ mice treated with $WAS^{-/-}$ nTreg cells was still very high. These data do not exclude that functional defects of $WAS^{-/-}$ nTreg cells may arise in vivo with different antigens and in different settings, such as those presented in the complementary study by Maillard et al. (48) using a different $WAS^{-/-}$ mouse strain. Indeed, $WAS^{-/-}$ nTreg cells displayed a defective in vivo suppressor activity toward colitis induced by transfer of WT CD45RB^{high} cells. $WAS^{-/-}$ nTreg cell defects may therefore be exacerbated during the development of spontaneous inflammatory bowel disease (42).

Collectively, our data suggest that in WASP deficiency the impaired suppressor activity of nTreg cells may be due to multiple defects, including intrinsic defects in activation and TGF-β production, as well as defects in other cell types, which therefore determine the extent of the impairment in homeostasis and peripheral tolerance.

Importantly, the role of WASP in regulating nTreg cell function was confirmed by our study in WAS patients. In seven WAS patients of different ages and with different disease severity, we found that the percentages of circulating CD4$^+$CD25$^+$FOXP3$^+$ nTreg cells were within or above normal range, excluding a selective defect in nTreg cell differentiation and production. Increased frequency of FOXP3$^+$ cells in the blood of two patients was attributed to an increase in the CD4$^+$CD25$^-$FOXP3$^+$ cell population, as seen in the thymuses and spleens of $WAS^{-/-}$ mice. This population has been proposed to be a pool of tissue-homing nTreg cells (9). Therefore, the increase in this cell subset might reflect a reduced ability of $WAS^{-/-}$ nTreg cells to migrate to peripheral nonlymphoid organs. The in vitro suppressor activity of WASP-deficient nTreg cells on proliferation was clearly impaired, both toward allogeneic and autologous effector T cells. nTreg cells from WAS patients (two out of two) also failed to suppress IFN-γ production by autologous effectors, whereas a residual regulatory activity toward IFN-γ production by allogeneic responder cells (two out of three patients) was observed. This is in line with a previous report showing that suppression of proliferation and cytokine production by nTreg cells isolated from patients with rheumatoid arthritis could occur independently (52).

Based on the results presented in this study, we hypothesize that one possible cause of autoimmunity and atopic disease in WAS patients is a functional defect of nTreg cells. Recent studies reported a compromised function of nTreg cells in several autoimmune diseases, including immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (50), multiple sclerosis (53), autoimmune polyglandular syndrome type II (54), rheumatoid arthritis (52), myasthenia gravis (55), and type 1 diabetes (56).

The similarities between our murine and human studies (normal steady-state distribution of nTreg cells in the periphery with defective suppressor activity) validate the $WAS^{-/-}$ murine model for further investigation of the contribution of nTreg cell defects to autoimmunity. It will be particularly interesting to study in this model how microbial triggers may act in concert with the functional defect in nTreg cells to lead to autoimmunity.

In conclusion, we show here that WASP is specifically required for the activation and suppressive function of CD4$^+$CD25$^+$FOXP3$^+$ nTreg cells of both human and murine origin, but not for their differentiation. We propose that the functional failure of nTreg cells contributes to the development of autoimmunity and atopic disease in WAS.

MATERIALS AND METHODS

Mice. C57BL/6 $WAS^{-/-}$ mice were provided by K.A. Siminovitch (Mt. Sinai Hospital, Toronto, Canada) (43). $WAS^{-/-}$-CD45.1 mice were generated in our animal facility. No spontaneous colitis was observed in these mouse models. WT C57BL/6 and WT C57BL/6-CD45.1 control mice were purchased from Charles River Laboratories. All procedures were performed according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute.

Patients and human cells. The detailed clinical status of the patients is reported in Table I. Peripheral blood samples from WAS patients and age-matched HDs were obtained following standard ethical procedures (including informed consent) and with the approval of the San Raffaele Scientific Institute Internal Review Board. PBMCs were purified on Ficoll gradient (Nycomed Pharma A/S).

Immunophenotype and immunofluorescence analysis. The following mAbs were used for surface staining of murine cells: anti-CD4 (RM4-5), anti-CD8 (53–6.7), anti-CD24 (M1/69), anti-CD25 (PC61), anti-CD45.2 (104), anti-CD45RB (16A), anti-CD62L (MEL14), and anti-CD69 (H1.2F3), all from BD Biosciences. Intracytoplasmic staining was performed using the following mAbs: anti-WASP (B-9; Santa Cruz Biotechnology, Inc.), anti–CTLA-4 (UC10-4F10-11; BD Biosciences), and anti-GITR (108619; R&D Systems). The staining with anti-Foxp3 mAbs (FJK-16s; eBioscience) was performed according to the manufacturer's instructions. For immunofluorescence staining, purified T cells were incubated for 20 min with Orange-CMTMR–loaded CD90$^-$ splenocytes as APCs (at a 1:1 cell ratio) in absence or presence of anti-CD3 mAbs (2C11; BD Biosciences). Cells were transferred onto Poly-L-lysine–coated glass slides, fixed/permeabilized, and stained with AlexaFluor^{633}-conjugated Phalloidin (Invitrogen) and anti-WASP antibodies (H-250; Santa Cruz Biotechnology, Inc.), followed by AlexaFluor^{488}-conjugated goat anti–rabbit antibodies. The samples were examined on a Carl Zeiss LSM 510 confocal microscope with an x63 Plan-Apochromat objective.

Human PBMCs were stained with anti-CD4 mAbs (SK3) and anti-CD25 mAbs (2A3), all from BD Biosciences. FOXP3 expression was evaluated using PHC101 mAbs from eBioscience.

nTreg cell activation assays. CD90$^+$CD25$^-$ effector T cells and CD4$^+$CD25$^+$ nTreg cells were purified from murine splenocytes by a combination of immunomagnetic beads (Miltenyi Biotec) and FACS sorting. Purity of CD4$^+$CD25$^+$ nTreg cells was $\geq$90% in all experiments. Cells were plated at 10^5 cells/well in the presence of the indicated dose of plate-bound anti-CD3 mAbs (17A2; BD Biosciences) either alone or in combination with either 1 μg/ml anti-CD28 mAbs (37.51; BD Biosciences) or 100 U/ml rhIL-2 (Chiron Corporation). Cell proliferation was measured after a 96-h stimulation by liquid scintillation counting. TGF-β secretion was measured by specific ELISA (R&D Systems) on supernatants conditioned for 96 h. The Bio-Plex technology using the Mouse Th1/Th2 panel (all from Bio-Rad Laboratories) was used to measure the levels of IL-10 and IL-5 (after a 96-h stimulation), and IL-2, IL-4, IFN-γ, and TNF-α (after a 48-h stimulation) in conditioned supernatants. TCR down-regulation was evaluated by FACS analysis on splenocytes previously stained with anti-CD4 and anti-CD25 mAbs, and subsequently stimulated with the indicated dose of plate-bound anti-CD3 mAbs for 5 h.

In vitro suppression assays. Suppression assays using murine cells were performed as follows: $CD90^+CD25^-$ effector T cells were plated at 10^5 cells per well and cocultured with 10^5 (1:1 effector T cell/nTreg cell ratio), 4×10^4 (1:0.4 effector T cell/nTreg cell ratio), or 1.6×10^4 (1:0.16 effector T cell/nTreg cell ratio) $CD4^+CD25^+$ nTreg cells in the presence of the indicated amount of plate-bound anti-CD3 mAbs. Cell proliferation was measured after a 96-h stimulation by liquid scintillation counting. Percentage of suppression was calculated as compared with effector T cells.

For suppression assays using WAS patients' cells, $CD4^+CD25^+$ nTreg cells and $CD4^+CD25^-$ effector T cells were isolated from PBMCs by FACS sorting. Cells from HDs were used as control: $CD4^+$ T cells were purified either by FACS sorting or, alternatively, by immunomagnetic beads. In both cases, the purity of nTreg cells ranged from 80 to 95%, and the purity of effector cells was ≥95%. Suppression assays with purified human nTreg cells were performed as follows: 5×10^4 $CD4^+CD25^-$ effector T cells were stimulated by CD3-depleted APCs (irradiated at 6,000 rad) and 1 μg/ml of soluble anti-CD3 mAbs (Orthoclone OKT3; Janssen-Cilag). Alternatively, when very low numbers of nTreg cells were recovered, 10^4 effector T cells were cocultured with nTreg cells and stimulated with beads coated with anti-CD3 and anti-CD28 mAbs (Dynal). For each lot of beads used, the optimal responder/bead ratio and the optimal culture time were carefully determined. Suppressive activity of nTreg cells was assessed by coculture of effector T cells with nTreg cells at different ratios. Proliferation was evaluated by liquid scintillation counting, and IFN-γ secretion was evaluated on supernatants by the human Th1/Th2 cytokine cytometric bead array system (BD Biosciences). Values of IFN-γ in the supernatants were normalized to a number of 2×10^5 cells.

In vivo modulation of OVA-specific immune response. nTreg cells from either WT or $WAS^{-/-}$ mice (carrying the CD45.2 allele) were injected i.v. ($1–1.5 \times 10^6$ per mouse) into WT or $WAS^{-/-}$ syngeneic mice. For tracking experiments, WT-CD45.1 and $WAS^{-/-}$-CD45.1 mice were used as recipients. 1 d later, mice were immunized by footpad injection of 100 μg OVA (grade V; Sigma-Aldrich) emulsified in CFA (Sigma-Aldrich). 7–10 d later, donor-derived cells were tracked in draining and nondraining LNs, blood, spleen, lungs, and liver by staining with anti-CD45.2 mAbs. In parallel, the in vitro recall response to OVA was tested as follows: total lymphocytes isolated from draining LNs were plated at 5×10^5 cells per well and cultured for 72 h with 5 μg/ml OVA. Cells were then pulsed with [^{3}H]thymidine, and cell proliferation was measured by liquid scintillation counting.

Statistical analysis. Where data followed a normal distribution (checked by Kolmogorov-Smirnov test), experimental groups were compared with a two-tailed Student's *t* test. Otherwise, a two-tailed Mann-Whitney test was used. p-values of <0.05 were considered significant.

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