Published in final edited form as:
Clin Immunol. 2007 July ; 124(1): 41–48.

Impaired in vitro regulatory T cell function associated with Wiskott-Aldrich syndrome

Marsilio Adriani[1,#], **Joseph Aoki**[2,#], **Reiko Horai**[2], **Angela M. Thornton**[3], **Akihiro Konno**[1], **Martha Kirby**[1], **Stacie M. Anderson**[1], **Richard M. Siegel**[4], **Fabio Candotti**[1], and **Pamela L. Schwartzberg**[2]

1*Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892*

2*Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892*

3*National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892*

4*National Institute of Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892*

Abstract

Wiskott-Aldrich syndrome (WAS) is a primary immunodeficiency characterized by the contradictory coexistence of impaired T-cell function and exaggerated T-cell-mediated pathology, including autoimmunity and eczema. WAS protein (WASp)-deficient mice are also immunodeficient and can develop autoimmune disease. Since defects in regulatory T-cells (Treg) are associated with autoimmunity, we examined the presence and function of these cells in WAS patients and WASp-deficient mice. We found that $CD4^+CD25^+FOXP3^+$ Treg cells can develop in the absence of WASp expression. However, Treg cells both from WASp-deficient mice and from four out of five WAS patients studied showed impaired in vitro suppressor function. In WASp-deficient mice, this defect could be partially rescued by pre-activation with IL-2, suggesting that inadequate cell activation may play a role in WASp-deficient Treg dysfunction. These findings may provide insights into the complex pathophysiology and paradoxical phenotypes of WAS and suggest new therapeutic modalities for autoimmunity in these patients.

INTRODUCTION

The Wiskott-Aldrich syndrome (WAS) is a rare X-linked primary immunodeficiency characterized by thrombocytopenia, eczema, recurrent infections and a high incidence of malignancy (1-3). A large proportion of WAS patients (40-70%) are also affected by at least one autoimmune disorder (4,5). The disorder is caused by mutations of *WAS*, the gene encoding the WAS protein, WASp, a key regulator of actin polymerization that is expressed in non-erythroid hematopoietic cells (2). T cells isolated from WASp-deficient mice and WAS patients show multiple defects including reduced TCR-induced proliferation and IL-2 production associated with impaired actin polymerization, defects which are likely to contribute to the immunodeficiency in WAS (6-9).

Corresponding author: Fabio Candotti, MD, National Human Genome Research Institute, National Institutes of Health, 49 Convent Dr., Blg. 49, Rm 3A20, Bethesda, MD 20892-4442, Tel: (301) 435 2944, Fax: (301) 480 3678, E-mail: fabio@nhgri.nih.gov
[#]These authors contributed equally to this work

A central question in understanding the pathophysiology of WAS is why immunodeficient patients develop symptoms suggestive of hyperactivation of immune compartments, including eczema and autoimmune diseases (5,10). It has recently been recognized that $CD4^+CD25^+$ Treg cells play an important role in the negative regulation of immune responses and the prevention of autoimmunity (11-13). These cells are non-responsive (anergic) to TCR stimulation and can suppress proliferation of $CD4^+CD25^-$ T target cells in culture. Importantly, mutations affecting FOXP3, a transcription factor required for the development of this lineage, lead to severe autoimmune disorders in humans and mice (14-19). Abnormal Treg function has also been demonstrated in several human autoimmune disorders, including type 1 diabetes, systemic lupus erythematosus and rheumatoid arthritis (12). We therefore hypothesized that abnormalities in Treg cells may contribute to the pathogenesis of autoimmune complications associated with WAS. To evaluate this question, we examined the presence and function of Treg cells from WASp-deficient mice and humans.

METHODS

Patients

Clinical and molecular characteristics of the WAS patients included in this study are listed in Supplementary Table 1. Mutation analysis of the WAS gene and flow cytometry analysis of WASp expression were performed as described (20). All human studies were approved by the NHGRI Institutional Review Board.

Mice

$Was^{-/Y}$ and $Was^{-/-}$ (129S6/SvEvTac-Was^{tm1Sbs}/J) (8) and control 129S6/SvEvTac mice were obtained from Jackson Laboratory and Taconic, respectively and maintained in sterile housing with sterile food and water. Mice were maintained and experiments performed according to NHGRI Animal Care and Use Committee guidelines. All mice used in these studies were between 6-10 weeks of age.

Cell culture reagents and antibodies

Mouse cells were cultured in RPMI-1640 (Invitrogen), 10% FCS, 100 U/ml penicillin, 100 μg/ml Streptomycin sulfate and 2 mM glutamine. Human cells were cultured in X-VIVO 20 (BioWhitaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts Inc., Woodland, CA), 100 U/ml penicillin, 100 μg/ml Streptomycin sulfate and 2 mM glutamine (all from Invitrogen, Carlsbad, CA).

Antibodies were from BD PharMingen (San Diego, CA), with the exception of anti-human CD25 (Immunotech, Marseille, France), anti-human-CD3 (Ortho Diagnostics, Raritan, NJ), and anti-human and mouse/rat FOXP3 (eBioscience, San Diego, CA).

Treg cell isolation

To obtain mouse Treg cells, $CD4^{hi}CD25^{hi}CD8^-$ and $CD4^{hi}CD25^-CD8^-$ cells were isolated either by cell sorting or AutoMACS (Miltenyi) purification from purified lymph node and splenic $CD4^+$ cells pooled from 7-10 mice per genotype, as described (21). Mouse accessory cells (APCs) were obtained from complement-mediated T-cell depleted splenocytes treated with 10 μg/ml mitomycin (Sigma-Aldrich, St. Louis, MO), for 3h at 37°C.

Human Treg cells were purified by sorting $CD4^+CD25^{hi}$ cells as previously described (22). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ) gradient centrifugation, then stained for 30 minutes at room temperature with anti-CD4-PerCP-Cy5.5, anti-CD25-PE, anti-CD14-APC and anti-CD32-APC in staining buffer (PBS + 2% FBS). $CD4^+$ cells were identified on FACSAria (BD

Bioscience, Bedford, MA) by gating on lymphocytes using forward and side scatter and excluding APC-positive cells. Based on their degree of CD25 expression, $CD4^+CD25^{hi}$ (Treg, top 1% cells), $CD4^+CD25^{lo}$ (activated cells), and $CD4^+CD25^-$ (target cells) were then isolated as previously reported (22). Human accessory cells were obtained by immunomagnetic depletion of $CD3^+$ cells using CD3-coated beads (Miltenyi Biotec Inc., Auburn, CA) from PBMC of healthy controls, followed by irradiation (3500 R).

In vitro suppression assays

Mouse $CD4^+CD25^+$ Treg suppression assays were performed in triplicate using 50,000 $CD4^+CD25^-$ target cells, 50,000 accessory cells and varying numbers of $CD4^+CD25^+$ Tregs in the presence of 0.5 μg/ml soluble anti-CD3 (2C11) as described (21). Proliferative characteristics of Treg cells were assessed by stimulation with anti-CD3 in the presence of accessory cells, but in the absence of $CD4^+CD25^-$ target cells. Alternatively, $CD4^+CD25^+$ Treg cells were pre-activated with anti-CD3 (1 μg/ml) + 50U IL-2 for three days (23) in the presence of equivalent numbers of accessory cells prior to washing to remove IL-2 and testing in suppression assays.

Suppression assays using human Treg cells were performed essentially as previously described (24). Briefly, $CD4^+CD25^-$ targets and/or $CD4^+CD25^{hi}$ Treg cells were stimulated with a combination of soluble anti-CD3 (OKT3; Ortho Diagnostics) and anti-CD28 (28.2 BD Pharmingen) antibodies (5 μg/ml each) in U-bottom 96-well plates in duplicate at a ratio 1:1 (3×10^3 cells/well each) in a final volume of 200 μl with 3×10^4 allogeneic accessory cells/well. After 5 days of culture, 100 μl of media was removed per well for IL-2 detection using anti-human-ELISA assay kit II (BD Bioscience) and one μCi of [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ) was added to each well for 16 h before harvesting.

RESULTS

Treg function is defective in *Was* knockout mice

To evaluate the requirement for WASp in $CD4^+CD25^+$ Treg cells, we first studied Treg numbers and function in WASp-deficient mice. Although spleens and lymph nodes from WASp-deficient mice had fewer $CD4^+CD25^+$ T cells on average compared to wild type (WT) animals, we found that these cells expressed normal levels of Foxp3 (Fig. 1A). Interestingly, we observed relatively normal numbers of $CD4^+Foxp3^+$ cells in spleens of WASp-deficient animals. Gating on these $CD4^+Foxp3^+$ cells revealed lower levels of CD25 expression than on WT $CD4^+Foxp3^+$ cells, similar to what observed in IL-2 knockout mice (25), and perhaps accounting for the decreased numbers of $CD4^+CD25^+$ cells observed. As expected, isolated $CD4^+CD25^+$ cells from either WT or WASp-deficient mice failed to proliferate in response to anti-CD3 stimulation in the presence of antigen presenting cells (Fig. 1B, “Treg” bars).

To determine whether these Treg cells exhibited suppressor function, isolated $CD4^+CD25^+$ cells from wildtype (WT) and WASp-deficient mice were cultured with WT $CD4^+CD25^-$ cells in an in vitro suppression assay. Since WASp-deficient T cells proliferate poorly in response to TCR stimulation in vitro (8,9), we used WT $CD4^+CD25^-$ cells as targets in these assays. $CD4^+CD25^+$ T cells from WT mice suppressed proliferation of WT $CD4^+CD25^-$ cells in a dose-dependent fashion. In contrast, WASp-deficient $CD4^+CD25^+$ Treg cells failed to effectively suppress target cell proliferation over a wide range of cell ratios, even at suppressor to target ratios of one or greater (Fig. 1B).

Preactivation with IL-2 can partially rescue defects in suppression

Although WASp-deficient T cells respond poorly to TCR stimulation in vitro, their proliferative responses can be partially rescued by addition of IL-2, particularly in the presence

of accessory cells (6,9). To investigate whether the defective suppressor function resulted from a failure of adequate cell activation, we prestimulated $CD4^+CD25^+$ Treg cells in the presence of anti-CD3 plus IL-2 and accessory cells for three days. It has been previously shown that $CD4^+CD25^+$ Treg cells can proliferate and maintain suppressor activity in these culture conditions (26). After prestimulation, we observed similar proliferation of WT and WASp-deficient $CD4^+CD25^+$ cells (p=0.13, Student t-test), despite their lack of proliferation in the absence of IL-2 (Fig. 1C versus 1B, "Treg" bars). Preactivation clearly improved the ability of WASp-deficient Treg cells to suppress WT target cell proliferation (Figure 1D). However, WASp-deficient Treg cells still showed an approximately 4-fold reduction in suppressor activity when compared to WT Treg cells. Thus, although WASp-deficient mice develop $CD4^+CD25^+Fox3P^+$ Tregs, these cells exhibit impaired suppressor activity in vitro.

$CD4^+CD25^+FOXP3^+$ T cells are present with the same frequency in healthy controls and WAS patients

To determine whether Treg cell function was similarly affected in WAS, we examined PBMC from 5 WAS patients (Table 1) and 5 healthy control subjects. Staining with anti-CD4 and anti-CD25, and anti-human FOXP3 again demonstrated similar frequencies of $CD4^+CD25^+$, $CD4^+FOXP3^+$ or $CD25^+FOXP3^+$ double positive cells in each group (Fig. 2A and Supplementary Fig. 1).

To further characterize these cells, WAS $CD4^+CD25^{hi}$ "regulatory", $CD4^+CD25^{lo}$ "activated" and $CD4^+CD25^-$ "target" cell populations were isolated by cell sorting as previously reported (22). Sorted $CD4^+CD25^{hi}$ cells represented 0.8-1.5% of the total $CD4^+CD25^+$ T cells. The majority of these cells expressed high levels of FOXP3 protein (Fig. 2B), whereas $CD4^+CD25^{lo}$ and $CD4^+CD25^-$ cells lacked FOXP3 expression (data not shown). $CD4^+CD25^{hi}$ cells showed similar scatter characteristics to $CD4^+CD25^-$ target cells, had minimal proliferation in response to either anti-CD3 plus anti-CD28 or PHA stimulation and lacked IL-2 production after stimulation (Fig. 3 and data not shown). In contrast, $CD4^+CD25^{lo}$ T cells were larger and both readily proliferated and produced IL-2 after stimulation (data not shown). Because in humans, FOXP3 can be transiently expressed in some activated $CD4^+$ cells (27), we examined CD127 expression on sorted populations, which is inversely correlated with Treg function (28,29). Importantly, the majority of the cells in the top ~1% of $CD4^+CD25^+$ cells (our $CD4^+CD25^{hi}$ population) were $CD127^{lo}$ (Supplementary Fig. 2). In contrast, sorted $CD4^+CD25^{lo}$ cells expressed high levels of CD127, as expected from activated cells. Together, these findings are consistent with the conclusion that the majority of the $CD4^+CD25^{hi}$ cells isolated from both WAS patients and healthy controls represent bona fide Treg cells.

WAS Treg cells exhibit impaired inhibitory function in vitro

To examine the suppression capability of Treg cells, $CD4^+CD25^{hi}$ cells and $CD4^+CD25^-$ target cells, were stimulated with anti-CD3 and anti-CD28 alone or mixed with each other in a 1:1 ratio in the presence of allogeneic accessory cells. $CD4^+CD25^{hi}$ Treg cells isolated from control subjects were anergic and suppressed proliferation when co-cultured with $CD4^+CD25^-$ target cells (Fig. 3). WAS $CD4^+CD25^{hi}$ Treg cells were also anergic to stimulation with either anti-CD3 and anti-CD28 or PHA (Fig. 3 and Supplementary Fig. 3). However, consistent with the proliferative defects of WASp-deficient T cells (30), these experimental conditions did not induce significant proliferation of WAS $CD4^+CD25^-$ cells (Fig. 3A), thus precluding the assessment of inhibitory effects of WAS $CD4^+CD25^{hi}$ Treg cells on autologous target cells.

To bypass these problems, we evaluated the inhibitory effects of WAS Treg cells on $CD4^+CD25^-$ target cells from allogeneic healthy controls. Treg cells from 4 of the 5 WAS patients studied showed decreased ability to suppress proliferation of control target cells. In

two cases (WAS6, WAS23), the addition of WAS Treg cells to allogeneic target cells resulted in a paradoxical enhancement of cell proliferation, as previously observed in other disorders associated with Treg cell dysfunction (31,32). Thus, in the majority of cases, $CD4^+CD25^+$ Tegs from patients with WAS also show impaired suppressor function.

DISCUSSION

Naturally occurring $CD4^+CD25^+FOXP3^+$ Treg cells are regarded as major players in mediating peripheral tolerance to autoantigens, and thus, can prevent the onset of autoimmune diseases. In humans, this is substantiated by the high incidence of autoimmunity in patients with genetic loss of FOXP3 and the associated immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (17-19). In addition, Treg defects have also been reported in patients affected with common autoimmune disorders, including rheumatoid arthritis, multiple sclerosis, psoriasis, and type 1 diabetes (32-35). Our data demonstrates that $CD4^+CD25^+$ Treg cells from WASp-deficient mice have marked defects in suppressor function in vitro. Interestingly, the defects we observe in WASp-deficient cells are somewhat more severe than those described in the very recent report by Humbalt-Baron and colleagues (36). Moreover, our work provides evidence that the same defect is present in patients with WAS. Indeed, $CD4^+CD25^+$ Tregs from four out of five WAS patients studied also had impaired capability to suppress in vitro proliferation of target T cells. Together, these data raise the possibility that abnormal Treg cell function may contribute to the pathogenesis of autoimmune complications associated with WAS.

It has recently been shown that, unlike murine cells, activated human $CD4^+CD25^-$ T cells can transiently express FOXP3. Whether or not these activated $FOXP3^+$ T cells have suppressor activity is a current area of debate (27,37). It has also been recently reported that expression of the IL-7 receptor α chain, CD127, can discriminate between activated $CD25^+CD127^+$ T cells and $CD25^+CD127^-$ regulatory T cells (28,29). Although we have not sorted for a CD127-negative population in $CD4^+CD25^{hi}FOXP3^+$ T cells from WAS patients and controls (due to the limiting numbers of $CD4^+CD25^+$ cells available), we have verified that our stringent selection for $CD4^+CD25^{hi}$ cells markedly decreases the fraction of $CD127^+$ cells among sorted populations. Moreover, sorted cells from healthy controls and WAS patients contain equal fractions of $CD127^+$ cells. In addition, WAS patients and controls in this study showed similar frequencies of $CD4^+DR^+$ cells (Supplementary Fig. 1), indicating that the WAS patients examined did not have increased numbers of activated $CD4^+$ lymphocytes. Finally, sorted Treg cells from WAS patients and healthy controls failed to proliferate when stimulated with anti-CD3 and anti-CD28 (Fig. 3) or PHA, which can induce some proliferation of WAS T cells (Supplementary Fig. 2), thus exhibiting anergic characteristics of true Treg cells.

The exception of normal Treg suppression activity by cells from patient WAS34, however, indicates that defective Treg function is not consistent in all WAS patients. It is possible that Treg activity may decline with time, following the reported age-dependent attrition of the T lymphocyte compartment in WAS (38,39). However, it can also be hypothesized that the defect of Treg function in WAS is not complete and may be modified by other factors. Indeed, we have seen that in vitro suppression can be partially rescued by pre-activation of murine Treg cells with IL-2. However, our observations of marked defects in suppression in Treg cells from WASp-deficient mice, which completely lack protein expression and are more genetically homogeneous, strongly support that WASp is required for full Treg suppressor function.

Although we do not yet understand the mechanism(s) responsible for the defective function of WAS Treg cells, it is likely that multiple factors may contribute. Treg cell suppression function depends on TCR stimulation (40) and requires direct cell-cell interaction (41). WASp deficiency is associated with defective TCR-mediated activation and impaired formation of

the immunological synapse (6-9,42-44), both of which may affect Treg cell suppression activity. Interestingly, the partial rescue of in vitro suppression defects by pre-activation of WASp-deficient Treg cells with IL-2, suggests that optimization of T cell activation may overcome the defect of WAS Treg cells. However, other factors may also contribute to impaired negative regulatory mechanisms in WAS, including the documented defect in T cell receptor downregulation (9), and decreased and delayed expression of CTLA-4 (45), an important T cell inhibitory receptor. We have further found that WAS-deficient murine $CD4^+$ T cells show impaired TCR-dependent restimulation-induced cell death (Nikolov et al., PLS, FC, RMS, manuscript submitted). The relative contribution of these potential mechanisms to the breakdown of peripheral tolerance in WAS patients remains to be established. Nevertheless, the demonstration of impaired suppressor function in Tregs from both WASp-deficient mice and WAS patients suggests that defective regulatory T cell function may be an important factor contributing to the immune dysregulation in WAS and suggests avenues for exploration of novel and improved forms of treatment for autoimmune complications of this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The Authors are indebted to the patients and their families and thankful to Dr. Robert Sokolic and Ms. Elizabeth Garabedian for excellent clinical care, and to Robin Handon for excellent animal husbandry. This work was funded in part by NHGRI, NIAID and NIAMS intramural funds.

REFERENCES

[1]. Aldrich RA, Steinberg AG, Campbell DC. Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhea. Pediatrics 1954;13:133–139. [PubMed: 13133561]

[2]. OchsHDThrasherAJThe Wiskott-Aldrich syndrome. J Allergy Clin Immunol2006117725738 quiz 739 [PubMed: 16630926]

[3]. Wiskott A. Familiarer, angeborener Morbus Werlhofii? Monatsschr Kinderheilkd 1937;68:212–216.

[4]. Dupuis-Girod S, Medioni J, Haddad E, Quartier P, Cavazzana-Calvo M, Le Deist F, de Saint Basile G, Delaunay J, Schwarz K, Casanova JL, Blanche S, Fischer A. Autoimmunity in Wiskott-Aldrich syndrome: risk factors, clinical features, and outcome in a single-center cohort of 55 patients. Pediatrics 2003;111:e622–627. [PubMed: 12728121]

[5]. Schurman SH, Candotti F. Autoimmunity in Wiskott-Aldrich syndrome. Curr Opin Rheumatol 2003;15:446–453. [PubMed: 12819473]

[6]. Molina IJ, Sancho J, Terhorst C, Rosen FS, Remold-O'Donnell E. T cells of patients with the Wiskott-Aldrich syndrome have a restricted defect in proliferative responses. J Immunol 1993;151:4383–4390. [PubMed: 8409409]

[7]. Gallego MD, Santamaria M, Pena J, Molina IJ. Defective actin reorganization and polymerization of Wiskott-Aldrich T cells in response to CD3-mediated stimulation. Blood 1997;90:3089–3097. [PubMed: 9376590]

[8]. Snapper SB, Rosen FS, Mizoguchi E, Cohen P, Khan W, Liu CH, Hagemann TL, Kwan SP, Ferrini R, Davidson L, Bhan AK, Alt FW. Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. Immunity 1998;9:81–91. [PubMed: 9697838]

[9]. Zhang J, Shehabeldin A, da Cruz LA, Butler J, Somani AK, McGavin M, Kozieradzki I, dos Santos AO, Nagy A, Grinstein S, Penninger JM, Siminovitch KA. Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. J Exp Med 1999;190:1329–1342. [PubMed: 10544204]

[10]. Arkwright PD, Abinun M, Cant AJ. Autoimmunity in human primary immunodeficiency diseases. Blood 2002;99:2694–2702. [PubMed: 11929755]

[11]. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2:389–400. [PubMed: 12093005]

[12]. Lan RY, Ansari AA, Lian ZX, Gershwin ME. Regulatory T cells: development, function and role in autoimmunity. Autoimmun Rev 2005;4:351–363. [PubMed: 16081026]

[13]. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005;6:345–352. [PubMed: 15785760]

[14]. HoriSNomuraTSakaguchiSControl of regulatory T cell development by the transcription factor Foxp3. Science200329910571061 Epub 2003 Jan 1059 [PubMed: 12522256]

[15]. KhattriRCoxTYasaykoSARamsdellFAn essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol20034337342 Epub 2003 Mar 2003 [PubMed: 12612581]

[16]. FontenotJDGavinMARudenskyAYFoxp3 programs the development and function of CD4+CD25 + regulatory T cells. Nat Immunol20034330336 Epub 2003 Mar 2003 [PubMed: 12612578]

[17]. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, Bowcock AM. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 2000;106:R75–81. [PubMed: 11120765]

[18]. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001;27:20–21. [PubMed: 11137993]

[19]. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 2001;27:18–20. [PubMed: 11137992]

[20]. Wada T, Schurman SH, Otsu M, Garabedian EK, Ochs HD, Nelson DL, Candotti F. Somatic mosaicism in Wiskott-Aldrich syndrome suggests in vivo reversion by a DNA slippage mechanism. Proc Natl Acad Sci U S A 2001;98:8697–8702. [PubMed: 11447283]

[21]. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998;188:287–296. [PubMed: 9670041]

[22]. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol 2001;167:1245–1253. [PubMed: 11466340]

[23]. Thornton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of CD4+CD25 + T cell suppressor function. Eur J Immunol 2004;34:366–376. [PubMed: 14768041]

[24]. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells. Clin Immunol 2005;115:10–18. [PubMed: 15870015]

[25]. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol 2005;6:1142–1151. [PubMed: 16227984]

[26]. Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. J Immunol 2004;172:6519–6523. [PubMed: 15153463]

[27]. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD, Rudensky AY. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci U S A 2006;103:6659–6664. [PubMed: 16617117]

[28]. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, Fazekas de St Groth B. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006;203:1693–1700. [PubMed: 16818676]

[29]. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, de St Groth BF, Clayberger C, Soper DM, Ziegler SF, Bluestone JA. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203:1701–1711. [PubMed: 16818678]

[30]. Molina IJ, Kenney DM, Rosen FS, Remold-O'Donnell E. T cell lines characterize events in the pathogenesis of the Wiskott-Aldrich syndrome. J Exp Med 1992;176:867–874. [PubMed: 1512549]

[31]. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006;108:253–261. [PubMed: 16537805]

[32]. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4 +CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004;199:971–979. [PubMed: 15067033]

[33]. Cao D, Malmstrom V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C. Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis. Eur J Immunol 2003;33:215–223. [PubMed: 12594850]

[34]. Sugiyama H, Gyulai R, Toichi E, Garaczi E, Shimada S, Stevens SR, McCormick TS, Cooper KD. Dysfunctional blood and target tissue CD4+CD25high regulatory T cells in psoriasis: mechanism underlying unrestrained pathogenic effector T cell proliferation. J Immunol 2005;174:164–173. [PubMed: 15611238]

[35]. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 2005;54:92–99. [PubMed: 15616015]

[36]. Humblet-Baron S, Sather B, Anover S, Becker-Herman S, Kasprowicz DJ, Khim S, Nguyen T, Hudkins-Loya K, Alpers CE, Ziegler SF, Ochs H, Torgerson T, Campbell DJ, Rawlings DJ. Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. J Clin Invest. 2007

[37]. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003;112:1437–1443. [PubMed: 14597769]

[38]. Ochs HD, Slichter SJ, Harker LA, Von Behrens WE, Clark RA, Wedgwood RJ. The Wiskott-Aldrich syndrome: studies of lymphocytes, granulocytes, and platelets. Blood 1980;55:243–252. [PubMed: 6444359]

[39]. Wada T, Schurman SH, Garabedian EK, Yachie A, Candotti F. Analysis of T-cell repertoire diversity in Wiskott-Aldrich syndrome. Blood 2005;106:3895–3897. [PubMed: 16091449]

[40]. Piccirillo CA, Thornton AM. Cornerstone of peripheral tolerance: naturally occurring CD4+CD25 + regulatory T cells. Trends Immunol 2004;25:374–380. [PubMed: 15207505]

[41]. Fehervari Z, Sakaguchi S. CD4+ Tregs and immune control. J Clin Invest 2004;114:1209–1217. [PubMed: 15520849]

[42]. Badour K, Zhang J, Shi F, McGavin MK, Rampersad V, Hardy LA, Field D, Siminovitch KA. The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. Immunity 2003;18:141–154. [PubMed: 12530983]

[43]. Dupre L, Aiuti A, Trifari S, Martino S, Saracco P, Bordignon C, Roncarolo MG. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. Immunity 2002;17:157–166. [PubMed: 12196287]

[44]. Huang W, Ochs HD, Dupont B, Vyas YM. The Wiskott-Aldrich syndrome protein regulates nuclear translocation of NFAT2 and NF-kappa B (RelA) independently of its role in filamentous actin polymerization and actin cytoskeletal rearrangement. J Immunol 2005;174:2602–2611. [PubMed: 15728466]

[45]. Aoki J, Konno A, Jankovic D, Cannons J, Candotti F, Schwartzberg PL. The role of Wiskott Aldrich syndrome protein in T helper cell function. Clin Immunol 2005:S249.

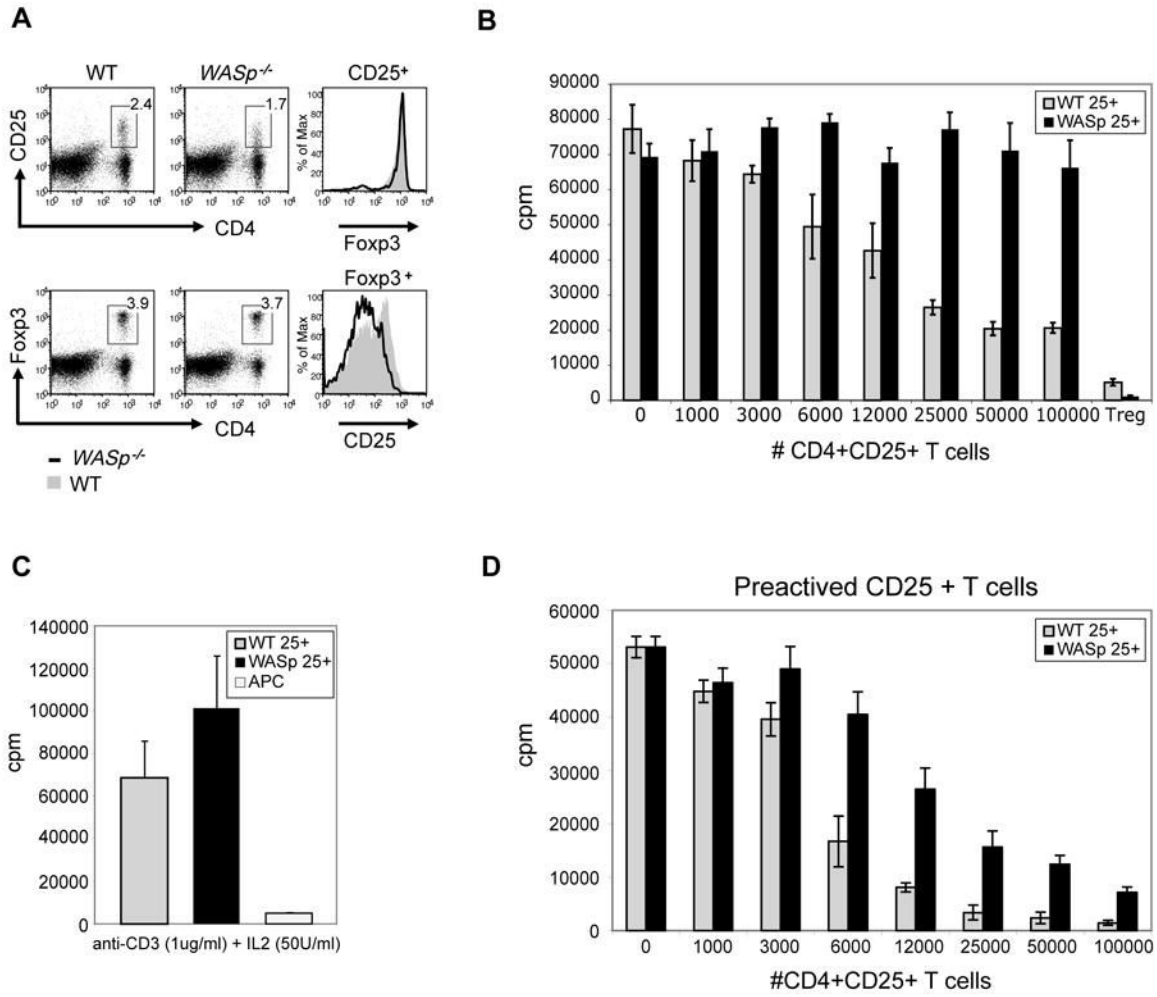

Figure 1. Impaired suppression function of $CD4^+CD25^+$ Treg cells from WASp-deficient mice

(A) WASp-deficient mice have similar numbers of $CD4^+Foxp3^+$ regulatory T cells, but these cells express lower levels of CD25. Flow cytometry of splenic cells are shown. (B) Freshly isolated WASp-deficient $CD4^+CD25^+$ Treg cells poorly suppress the proliferation of WT $CD4^+CD25^-$ target cells. WT $CD4^+CD25^-$ target cells (50,000/well) were plated with accessory cells and co-cultured in triplicate with the indicated numbers of freshly isolated WT or $WASp^{-/-}$ $CD4^+CD25^+$ Treg cells in the presence of anti-CD3. Treg sample refers to $CD4^+CD25^+$ T cells cultured with accessory cells in the presence of anti-CD3. Cell proliferation was assessed at the end of a 3-day culture period by [^{3}H]-thymidine incorporation and is presented as the mean ± SEM of triplicate cultures. Data is representative of three independent experiments. (C) [^{3}H]-thymidine incorporation by WASp-deficient and WT Treg cells following 3-day pre-activation (stimulation with anti-CD3 + IL-2). (D) Pre-activated WASp-deficient Treg cells show reduced suppression activity against the proliferation of WT $CD4^+CD25^-$ target cells. Pre-activated $CD4^+CD25^+$ Treg cells were plated and cultured as in Figure 2B. Cell proliferation was assessed by [^{3}H]-thymidine incorporation after 3-days of culture, as above. Data is the mean of triplicate cultures ± SEM and is representative of two independent experiments. Average proliferation of $CD4^+CD25^+$ was less than 220 cpm.

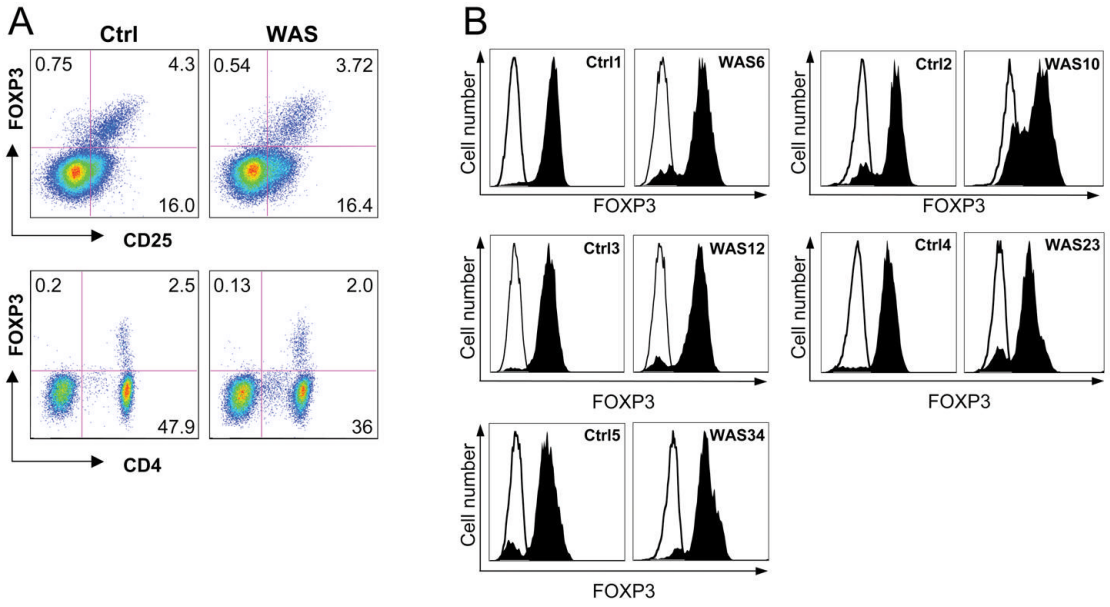

Figure 2. Phenotype of $CD4^{+}CD25^{hi}$ Treg cells in healthy controls and WAS patients

(A) Prevalence of $CD4^{+}FOXP3^{+}$ or $CD25^{+}FOXP3^{+}$ cells in from healthy control (Ctrl) and WAS $CD14^{-}CD32^{-}$ lymphocytes. Quadrant markers were set based on staining with the isotype matched control mAbs and percentage of cells in each quadrant are indicated. Data shown are representative of 5 different experiments. (B) FOXP3 expression in healthy control and WAS $CD4^{+}CD25^{hi}$ Tregs were characterized by intracellular staining with anti-human FOXP3-APC antibody. Open histograms represent isotype controls; solid histograms represent FOXP3-specific staining.

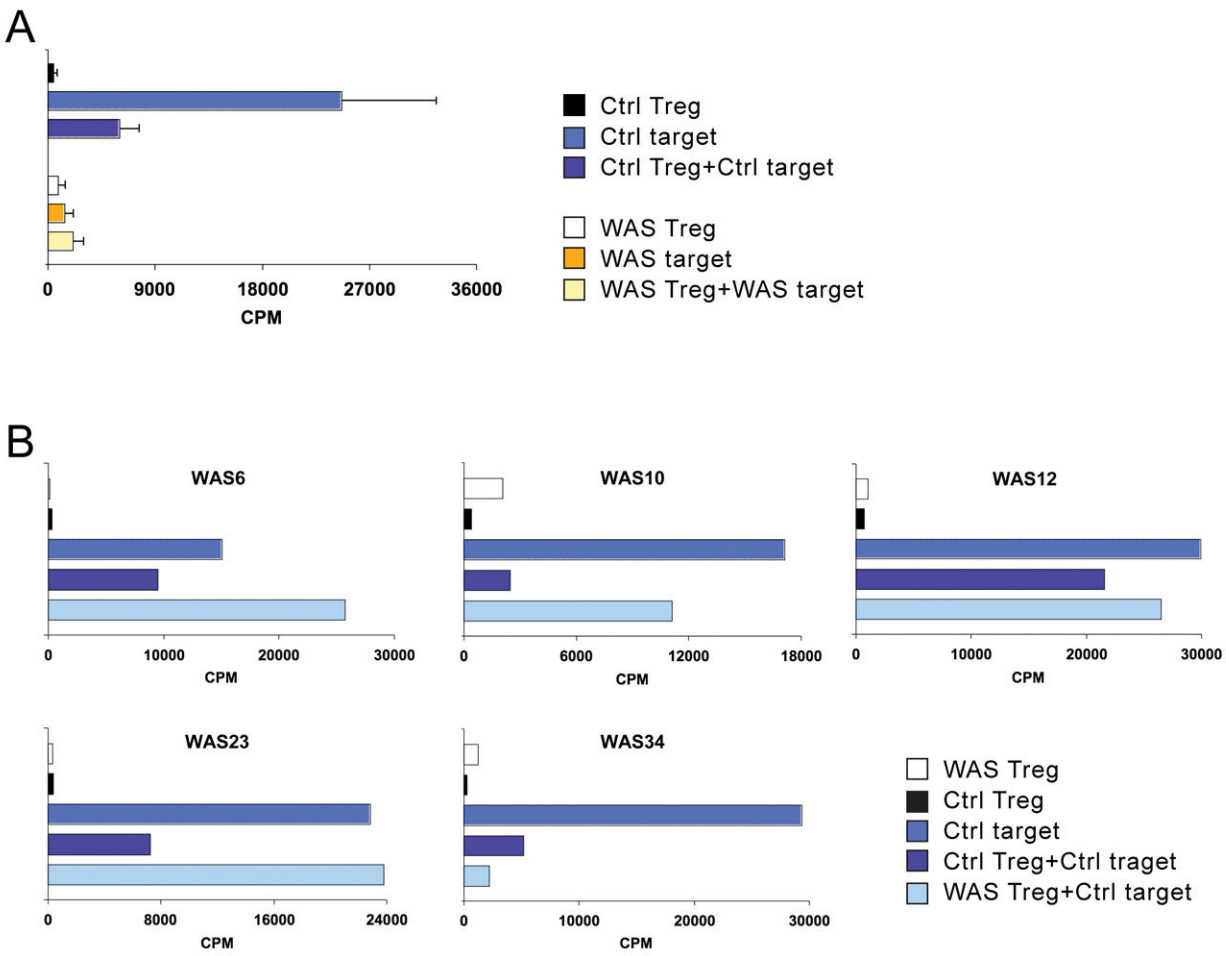

Figure 3. Treg suppression assays after stimulation with anti-CD3 plus anti-CD28 antibodies

(A) Treg suppression activity on autologous target cells. Control (Ctrl) Treg cells and Ctrl target cells were plated alone or co-cultured in a 1:1 ratio and stimulated with anti-CD3 + anti-CD28 in the presence of accessory cells. Similar assays were performed with WAS Treg and WAS target cells. Cell proliferation assessed as incorporation of [^{3}H]-thymidine at the end of 5 days of culture is shown as counts per minute (CPM). Data are the mean ± SD of 5 independent experiments using samples from 5 different healthy controls and the 5 WAS patients described in Supplementary Table 1. (B) Ctrl Treg cells, WAS Treg cells, and Ctrl target cells were plated alone and stimulated with anti-CD3 + anti-CD28 in the presence of accessory cells. Ctrl Treg and WAS Treg were also co-cultured with Ctrl target cells in a 1:1 ratio and stimulated as above. Cell proliferation was assessed as incorporation of [^{3}H]-thymidine at the end of 5 days of culture and is shown as counts per minute (CPM). Data shown are mean values of duplicate cultures.

Table 1

Patient Characteristics

Subject	Age (years)	Mutation	WASP expression	Lymphocytes/NL ($\times 10^9$/L)	Autoimmunity signs/symptoms	Score*
WAS6	24	C290T/ Arg66Cys	Reduced	1.74/0.46-4.7[a]	Proteinuria	3
WAS10	23	1305insG/fs stop aa494	Absent	0.88/0.46-4.7[a]	Relapsing polychondritis	5
WAS12	27	G389A/ Gly119Arg	Reduced	1.89/0.46-4.7[a]	Positive LA Arthralgias	2
WAS23	2	G291A/ Arg66His	Reduced	3.72/1.7-6.9[b]	None	2-3
WAS34	4	C995T/ Arg321Stop	Absent	1.74/1.7-6.9[b]	None	3

NL: normal limits for age (a: adult reference values from NIH Clinical Center Department of Laboratory Medicine; b: pediatric reference values from J. Pediatr. 1997, 130:388)

LA: lupus anticoagulant

* as described in Zhu et al.6