Defective Th1 Cytokine Gene Transcription in CD4+ and CD8+ T Cells from Wiskott-Aldrich Syndrome Patients

Sara Trifari, Giovanni Sitia, Alessandro Aiuti, Samantha Scaramuzza, Francesco Marangoni, Luca G. Guidotti, Silvana Martino, Paola Saracco, Luigi D. Notarangelo, Maria-Grazia Roncarolo and Loïc Dupré

*J Immunol* 2006; 177:7451-7461; doi: 10.4049/jimmunol.177.10.7451
http://www.jimmunol.org/content/177/10/7451

**References** This article cites 58 articles, 30 of which you can access for free at:
http://www.jimmunol.org/content/177/10/7451.full#ref-list-1

**Subscription** Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Defective Th1 Cytokine Gene Transcription in CD4+ and CD8+ T Cells from Wiskott-Aldrich Syndrome Patients

Sara Trifari,* Giovanni Sitia,† Alessandro Aiuti,*, Samantha Scaramuzza,*, Francesco Marangoni,++, Luca G. Guidotti,++, Silvana Martinò,⁷ Paola Saracco,⁷ Luigi D. Notarangelo,‖ Maria-Grazia Roncarolo,²*‡ and Loïc Duprè*²

Wiskott-Aldrich syndrome (WAS) protein (WASP) plays a key role in TCR-mediated activation and immunological synapse formation. However, the effects of WASP deficiency on effector functions of human CD4+ and CD8+ T cells remain to be determined. In this study, we report that TCR/CD28-driven proliferation and secretion of IL-2, IFN-γ, and TNF-α are strongly reduced in CD8+ T cells from WAS patients, compared with healthy donor CD8+ T cells. Furthermore, WAS CD4+ T cells secrete low levels of IL-2 and fail to produce IFN-γ and TNF-α, while the production of IL-4, IL-5, and IL-10 is only minimally affected. Defective IL-2 and IFN-γ production persists after culture of naive WAS CD4+ T cells in Th1-polarizing conditions. The defect in Th1 cytokine production by WAS CD4+ and CD8+ T cells is also present at the transcriptional level, as shown by reduced IL-2 and IFN-γ mRNA transcripts after TCR/CD28 triggering. The reduced transcription of Th1 cytokine genes in WAS CD4+ T cells is associated with a defective induction of T-bet mRNA and a reduction in the early nuclear recruitment of NFAT-1, while the defective activation of WAS CD4+ T cells correlates with reduced nuclear recruitment of both NFAT-1 and NFAT-2. Together, our data indicate that WASP regulates the transcriptional activation of T cells and is required specifically for Th1 cytokine production. The Journal of Immunology, 2006, 177: 7451–7461.

Wiskott-Aldrich syndrome (WAS) protein (WASP) is expressed in cells of hemopoietic origin and is involved in actin cytoskeleton remodeling, thereby playing a key role in chemotaxis, adhesion, phagocytosis, and traffic of various hemopoietic cells (1). In addition, WASP controls the assembly of synaptic structures among different cells of the immune system (2, 3). In T cells, upon triggering of TCR and costimulatory molecules, WASP is recruited to the immunological synapse and is required for its organization (4, 5). Regulation of WASP localization is under the control of WASP-interacting protein and Nck adaptor protein/SH2-domain containing leucocyte protein of 76 kDa (6–8), while its activation status is modulated by binding to the small Rho GTPase Cdc42 and by phosphorylation (9–13). The active form of WASP induces actin nucleation through binding to actin-related protein 2/3 (14). The molecular mechanisms by which WASP integrates multiple signals to promote actin polymerization and control immunological synapse assembly have been extensively studied. However, the role of WASP in controlling downstream signaling and, more in general, T cell functions is still largely unknown.

Mutations in the WAS gene in humans may lead to different clinical phenotypes, ranging from WAS (15), characterized by microthrombocytopenia, eczema, immunodeficiency, autoimmune disorders, and high incidence of lymphoid malignancies (1), to X-linked thrombocytopenia (XLT), a milder form of the disease with minimal or no immunodeficiency (16, 17). A strong correlation between expression of WASP in lymphocytes and clinical phenotype has been recently reported in patients carrying WAS gene mutations (18, 19). Although WASP deficiency affects the functions of both lymphoid and myeloid cells, results obtained from the follow-up of WAS patients who underwent HLA-matched hemopoietic cell transplant, and from the analysis of WAS patients with spontaneous genetic reversion, indicate that WASP expression confers a selective growth advantage to T cells only (20, 21). Consistently, it has been shown that WAS patients have a reduction in the numbers of circulating T lymphocytes, especially within the naive compartment (22). WAS patients suffer from recurrent infections caused by encapsulated bacteria, viruses, and fungi (19), suggesting an impairment in cellular-mediated immunity. Moreover, they have a high susceptibility to develop lymphomas (19) which could be due to poor immune surveillance. However, T cell functions are not completely abrogated in WAS patients, as illustrated by the high frequency of T cell-mediated autoimmune manifestations (23). T cells from WAS patients proliferate poorly, secrete low levels of IL-2 in response to anti-CD3 mAb and display abnormal cell morphology (4, 24, 25). Defective secretion of IL-2, IFN-γ, TNF-α, and IL-4 in response to TCR or TCR/CD28-mediated stimulation has been shown in WASP knockout mice (26–29). Although proximal TCR signaling is not significantly affected in T cells from WASP knockout mice (26), WASP seems to be required for downstream events, including activation of NFAT-1 (NFAT-p), NFAT-2 (NFAT-c), and expression of Fos (13, 30, 31). Similar results have been obtained in NK

Copyright © 2006 by The American Association of Immunologists, Inc.
cells from WAS patients, in which a time-restricted defect in the nuclear translocation of NFAT-2 and RelA (NF-κB) was observed in response to ligation of the NKP46R (32).

In this study, we investigated the effector functions of WASP-deficient CD4⁺ and CD8⁺ T cell lines generated from WAS patients. We show that, in response to TCR/CD28-mediated activation, WAS CD4⁺ and CD8⁺ T cells have a selective impairment in Th1 cytokine production, which is due to defective gene transcription. This defect is associated, in WAS CD4⁺ T cells, to a reduction in T-bet mRNA expression and in the early nuclear recruitment of NFAT-1. In WAS CD8⁺ T cells, the block in cytokine production correlates with reduced nuclear levels of both NFAT-1 and NFAT-2. These results suggest that WASP may have a predominant role in cellular-mediated immunity by controlling Th1 cytokine production at the transcriptional level.

**Materials and Methods**

**Patients and cells**

The patients studied here have been previously described (4, 16, 18). Patient W1 was diagnosed as WAS (clinical score 4, according to Zhu et al. (33)), on the basis of clinical phenotype (eczema, thrombocytopenia, recurrent HSV infections, candidiasis, pneumonia) and WAS gene mutation. He has been often treated with antibiotic prophylaxis and i.v. Ig infusions and his clinical conditions are stable. Patient W2 was diagnosed as WAS (score 5) based on clinical symptoms and molecular analysis of the WAS gene. At the age of 6 years, he underwent bone marrow transplant from a matched unrelated donor. He died in the first year following bone marrow transplant due to CMV infection. Patient X6 was diagnosed as XLT (disease score 0.5). He has thrombocytopenia and transient eczema but has not developed severe infections. Patient W8 was diagnosed as WAS. He developed thrombocytopenia and mild eczema, with recurrent otitis media (disease score 3). He died of intracranial hemorrhage at the age of 3 years. Patient W9 was diagnosed as WAS, thrombocytopenia, eczema, thrombocytopenia, episodes of bloody diarrhea, and airways infections (disease score 3). Blood samples from WAS patients and healthy donors (HD) were obtained according to standard ethical procedures and with the approval of the concerned internal review boards. Untransformed polyclonal T cell lines were generated from PBMC of WAS patients and HDs as previously described (4). Briefly, PBMC were purified from peripheral blood on Lymphoprep (Nycomed Pharma) gradient and seeded at a concentration of 5 × 10⁵ cells/ml. Cells were stimulated in the presence of 1 × 10⁶/ml allogeneic PBMC (x-ray irradiated at 6.000 rad) and 1 × 10⁶/ml EBV-transformed B cell line JY (x-ray irradiated at 10.000 rad), 1 μg/ml PHA, and 100 IU/ml recombinant human IL-2 (Chiron). Cells were cultured in IMDM (Cambrex Bio Science) supplemented with 10% YSSEL medium (Dyaclone), recombinant human IL-2 (Chiron), and penicillin/streptomycin (Bristol-Myers Squibb). T cells were restimulated as described every 2 wk. CD4⁺ and CD8⁺ T cell subsets were purified by positive selection from established T cell lines, using anti-CD4 or anti-CD8 microbeads, respectively (Miltenyi Biotec) according to the manufacturer’s instructions (purity was >95%).

**WASP expression**

Cell extracts were prepared from 1 × 10⁶ resting T cells. Briefly, T cells were washed in PBS and resuspended in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40), supplemented with protease inhibitor mixture (Sigma Aldrich). After 30 min on ice, lysates were cleared by centrifugation and an aliquot of supernatants was used for determining protein concentration with the BCA protein assay (Pierce). Equal amount of proteins were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and WASP was detected by anti-WASP polyclonal Ab H-250 (Santa Cruz Biotechnology) followed by secondary HRP-conjugated anti-mouse Abs. Control of protein loading was performed by hybridizing the same membranes with an anti-G3PDH mAb (Chemicon International). Detection was performed using the ECL detection system (Amersham Biosciences).

**Differentiation of Th1 cells**

Naive CD4⁺ T cells were purified from PBMC of W1 and of two age-matched HDs by negative selection, using the CD4⁺ T cells isolation kit according to the manufacturer’s instructions (Miltenyi Biotec). Naive CD4⁺ T cells were obtained by depleting CD45RO⁺ cells with anti-CD45RO Miltenyi beads (Miltenyi Biotech), according to the manufacturer’s instructions (purity was: 85-95%). Naive CD4⁺ T cells were activated by coculture with murine L cells, stably transfectected with human (h) CD32 (FcγRII), hCD58 (LFA-3), and hCD80 (34). Briefly, L cells were trypsinized and x-ray irradiated (7000 rad). Following washing, cells were seeded at 4 × 10⁵ cells/well of a 24-well plate in IMDM supplemented with 10% FBS, penicillin/streptomycin, and 100 ng/ml anti-CD3 mAb (Orthoclone OKT3; Janssen-Cilag). After adhesion of L cells, 4 × 10⁵ naive CD4⁺ T cells were plated in either nonpolarizing Th0 conditions, or in the presence 10 ng/ml recombinant human IL-12 (R&D Systems), and 200 ng/ml anti-hiL-4 mAb (BD Biosciences Pharmingen) for Th1 polarization. At day 3, 40 IU/ml IL-2 was added to all culture conditions. After 1 wk, cells were restimulated with either 10 μg/ml plate-bound anti-CD3 mAb plus 1 μg/ml soluble anti-CD28 mAb, or with 10 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA; Calbiochem) plus 500 ng/ml ionomycin (Calbiochem) and analyzed for the profile of cytokine production.

**T cell stimulation, proliferation, and cytokine secretion**

Resting T cells (9–14 days after stimulation) were starved overnight without IL-2, harvested, and seeded at 1 × 10⁷ cells/well in a round-bottom 96-well plate. Latex beads (2 × 10⁵; Interfacial Dynamic), coated with the indicated amount of anti-CD3 and anti-CD28 mAb (BD Biosciences Pharmingen), as previously described (4), were added to the cells. Overnight, T cells were cultured in medium with 200 μl/well (bead:cell ratio = 2:1). Alternatively, T cells were stimulated with plastic-bound anti-CD3 plus soluble anti-CD28 mAb. In this case, plates were coated overnight with anti-CD3 mAb in 0.1 M Tris (pH 9.5), and washed in PBS. T cells were plated at an initial density of 1 × 10⁶ cells/well in a final volume of 200 μl in the presence of 10 μg/ml soluble anti-CD28 mAb.

T cell proliferation was analyzed after 72 h of stimulation, by a 16-h pulse with 1 μCi/well [³H]thymidine (Amersham Biosciences) followed by harvesting with a 96-well plate harvester (Tomtec) and counting by liquid scintillation. All experiments were performed in duplicate or triplicate wells. To measure cytokine secretion, supernatants of resting and activated T cells were collected at different time points (18 h for IL-2 and 48 h for the other cytokines). For detection of IL-2, IFN-γ, IL-4, IL-5, IL-10, and TNF-α, capture ELISA was performed according to the manufacturer’s instructions (BD Biosciences Pharmingen).

**Levels of intracellular cytokines**

Resting T cells (1 × 10⁵/ml) were stimulated either with beads coated with 1 μg/ml anti-CD3 mAb plus 10 μg/ml anti-CD28 mAb or with 10 ng/ml TPA plus 500 ng/ml ionomycin in complete medium. Upon initiation of the experiment, plates were washed and reconstituted for 2 min at 37°C. After additional 3 h, T cells were collected and fixed in 2% formaldehyde. After fixation, T cells were permeabilized by 10 min incubation in blocking buffer (PBS 0.3% BSA, 0.1% NaN₃). Briefly, L cells were coated with PE-labeled anti-CD3, anti-CD28 mAb, and FITC-labeled anti-CD25 mAb. Permeabilized T cells were incubated in blocking buffer and stained with FITC-labeled anti-CD8 mAb, PE-labeled anti-CD45RO mAb, PE-labeled anti-hiL-2, and RTIC-labeled anti-hIFN-γ (BD Biosciences Pharmingen). After washing, cells were analyzed on a FACScan and data were analyzed with CellQuest software (BD Biosciences).

**Cytokine mRNA expression**

Resting T cells were stimulated by 1 μg/ml immobilized anti-CD3 mAb plus 10 μg/ml soluble anti-CD28 mAb, or by 10 ng/ml TPA plus 500 ng/ml ionomycin for 4 h of incubation at 37°C, in a 5% CO₂ atmosphere. T cells were collected and RNA was extracted as previously described (35). Total RNA was extracted from either CD4⁺ or CD8⁺ T cells and the levels of mRNA of IFN-γ, IL-2, IL-4, IL-5, IL-10, and anti-hiL-2, and RTIC-labeled anti-hIFN-γ (BD Biosciences Pharmingen). After washing, cells were analyzed on a FACScan and data were analyzed with CellQuest software (BD Biosciences).
To measure T-bet and GATA-3 mRNA levels, CD4+ T cell lines from WAS patients and HDs were stimulated with immobilized anti-CD3 mAb (0.1–1 μg/ml) plus 10 μg/ml soluble anti-CD28 mAb for 4 h. Total RNA was extracted from 1 × 10^6 cells using Eurozol (Euroclone) and RNA was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s instructions. T-bet and GATA-3 mRNA species were quantified using the Assay on Demand kit (Applied Biosystems) and TaqMan Master Mix (Applied Biosystems). Each sample was analyzed in duplicates and the relative expression of T-bet and GATA-3 was defined by calculating the ΔΔct threshold, with respect to the housekeeping gene HPRT, which was run in the same tube.

**NFAT and Fos nuclear levels**

To detect nuclear levels of NFAT-1, NFAT-2, and Fos family members, Western blotting, 12 person Western blotting, 12 persons (Fig. 1, A and B). FACS analysis showed that T cells from the XLT patient were uniformly WASPdim (data not shown). Upon stimulation with beads coated with a fixed dose of anti-CD28 mAb and increasing doses of anti-CD3 mAb, both CD4+ and CD8+ T cells from WAS patients showed defective proliferation, irrespective of the dose of anti-CD3 mAb used. However, the defect was more evident at low anti-CD3 mAb doses. Interestingly, cells from the XLT patient displayed an intermediate proliferative capacity (Fig. 1, C). In patients X6, W1, and W2, the proliferative defect was reproducibly more severe in CD8+ T cells, compared with CD4+ T cells, while CD4+ and CD8+ T cells from patients W8 and W12 showed a comparable defect of proliferation (Fig. 1, C and D). Together, these results indicate that both CD4+ and CD8+ T cells require WASP for TCR/CD28-induced proliferation and polyclonal Ab, which recognizes c-Fos, Fos-B, and Fra-1/2 (Santa Cruz Biotechnology). Protein normalization and quality of cell fractionation were assessed by reprobing the same nitrocellulose membranes with an anti-Sp1 polyclonal Ab (Santa Cruz Biotechnology) for nuclear extracts, and with an anti-G3PDH mAb (Chemicon International) for cytosolic extracts. Densitometric analysis was performed with ImageQuant software. Values corresponding to unstimulated cells were subtracted.

**Results**

**WASP is required for proliferation of CD4+ and CD8+ human T cells after TCR/CD28-mediated stimulation**

To investigate the role of WASP in the proliferative response of CD4+ and CD8+ T cells, we established polyclonal T cell lines from four WAS patients and one XLT patient (Table 1), which were analyzed for WASP expression. CD4+ and CD8+ T cells from all WAS patients lacked WASP expression, while CD4+ and CD8+ T cells from the XLT patient had residual WASP expression (Fig. 1, A and B). FACS analysis showed that T cells from the XLT patient were uniformly WASPdim (data not shown). Upon stimulation with beads coated with a fixed dose of anti-CD28 mAb and increasing doses of anti-CD3 mAb, both CD4+ and CD8+ T cells from WAS patients showed defective proliferation, irrespective of the dose of anti-CD3 mAb used. However, the defect was more evident at low anti-CD3 mAb doses. Interestingly, cells from the XLT patient displayed an intermediate proliferative capacity (Fig. 1, C and D). In patients X6, W1, and W2, the proliferative defect was reproducibly more severe in CD8+ T cells, compared with CD4+ T cells, while CD4+ and CD8+ T cells from patients W8 and W12 showed a comparable defect of proliferation (Fig. 1, C and D). Together, these results indicate that both CD4+ and CD8+ T cells require WASP for TCR/CD28-induced proliferation and polyclonal Ab, which recognizes c-Fos, Fos-B, and Fra-1/2 (Santa Cruz Biotechnology). Protein normalization and quality of cell fractionation were assessed by reprobing the same nitrocellulose membranes with an anti-Sp1 polyclonal Ab (Santa Cruz Biotechnology) for nuclear extracts, and with an anti-G3PDH mAb (Chemicon International) for cytosolic extracts. Densitometric analysis was performed with ImageQuant software. Values corresponding to unstimulated cells were subtracted.

**Statistical analysis**

Each experiment of proliferation, cytokine secretion, and T-bet/GATA-3 mRNA expression was performed in duplicate or triplicate. Analysis of statistically significant differences between each patient and the HDs was performed on a total of three to six independent experiments, using either the nonparametric two-tailed Wilcoxon rank sum test or the Wilcoxon signed rank test as indicated. Values of p < 0.05 were considered significant. Statistical analysis of NFAT-1/NFAT-2 and Fos nuclear levels in WAS patients vs control cells were performed on three to five independent experiments using the two-tailed Wilcoxon rank sum test.

**FIGURE 1. WASP expression and TCR-driven proliferation in T cell lines from WAS and XLT patients.** WASP expression was measured by Western blot in resting CD4+ T cell lines (A) and CD8+ T cell lines (B) from WAS (W1, W2, W8, and W12) and XLT (X6) patients, and from HD1 and HD2. Proliferation of CD4+ T cell lines (C) and CD8+ T cell lines (D) from HDs (mean ± SD of 4) and WAS/XLT patients was measured by [3H]thymidine incorporation, after 72-h stimulation with beads coated with the indicated doses of anti-CD3 mAb plus 10 μg/ml anti-CD28 mAb. Values represent mean of duplicate cultures and are representative of one of three independent experiments.
suggest that the levels of WASP expression correlate with the ability of CD4⁺ and CD8⁺ T cells to respond to TCR/CD28-mediated stimulation.

Defective Th1 cytokine production in WAS CD4⁺ T cells
Defective proliferation of WAS T cells could be due, at least in part, to suboptimal IL-2 secretion. To investigate this possibility and to further analyze the cytokine profile of WAS T cell lines, secretion of different cytokines was measured after stimulation with anti-CD3 plus anti-CD28 mAb-coated beads. In WAS CD4⁺ T cells, IL-2 secretion was below the normal range, but not abolished (Fig. 2A, p < 0.05). Interestingly, the more severe clinical phenotype of patient W2 corresponded to a more severe impairment in IL-2 secretion, compared with patient W1. Secretion of IFN-γ and TNF-α was severely compromised in both patients (Fig. 2A, p < 0.01). In contrast, secretion of IL-4, IL-5, and IL-10 did not differ significantly between patient W1 and control CD4⁺ T cells (Fig. 2A). The secretion of IL-5 by W2 CD4⁺ T cells was slightly defective (p < 0.05), while secretion of both IL-4 and IL-10 was normal (Fig. 2A). Stimulation with TPA/ionomycin induced the secretion of comparable levels of Th1 and Th2 cytokines by WAS and control CD4⁺ T cells (Fig. 2A).

To further confirm the bias in the cytokine profile of WAS CD4⁺ T cells, production of IL-2, IFN-γ, and IL-4 was measured by intracellular staining in CD4⁺ T cell lines from four WAS patients and one XLT patient. As shown in Fig. 2B, upon TCR/CD28 triggering, the percentages of CD4⁺ T cells which produced IL-2 and/or IFN-γ were strongly reduced in all WAS patients, with respect to HDs (range of IL-2⁺ cells in HDs CD4⁺ T cells activated with TCR/CD28, n = 5, was 28–44%; range of IFN-γ⁺ cells was 17–30%). In particular, in WAS patients, the population of cells producing both IL-2 and IFN-γ was the most affected.
Interestingly, CD4^+ T cells from the XLT patient produced normal levels of IL-2, while IFN-γ production remained below the normal range. Stimulation with TPA and ionomycin induced robust production of IL-2 and IFN-γ in CD4^+ T cells from both WAS patients and HDs. IL-4 production by WAS CD4^+ T cells was considerably higher than IL-2 and IFN-γ production. Among WAS patients, percentages of cells producing IL-4 only, were just below normal range (range of cells producing only IL-4 in HDs, \( n = 5 \), was 22–38%). However, the percentage of cells producing both IL-4 and IFN-γ was strongly reduced in WAS CD4^+ T cells, compared with control cells. Together, these data demonstrate that in normal range (range of cells producing only IL-4 in HDs, \( n = 5 \), was 22–38%). However, the percentage of cells producing both IL-4 and IFN-γ was strongly reduced in WAS CD4^+ T cells, compared with control cells. The defect was mostly evident in the subset of IL-2^+/IFN-γ^+ CD4^+ T cells. Stimulation with TPA/ionomycin induced similar levels of cytokine production in W1 and control Th1 cells. These data show that, although WASP-deficient CD4^+ T cells can undergo Th1 differentiation in vitro, they retain a defect in the production of IFN-γ and IL-2 in response to TCR/CD28-mediated signals.

**Defective cytokine production in WAS CD8^+ T cells**

We next investigated the cytokine production and secretion by WAS and XLT CD8^+ T cell lines, generated in parallel to the CD4^+ T cell lines. CD8^+ T cells from patients W1 and W2 showed a complete block in the secretion of IFN-γ and IL-2 in response to TCR/CD28-mediated signals.
levels of IL-2, IFN-γ, and TNF-α by WAS and control CD8+ T cells (Fig. 4A).

IL-5 secretion by WAS CD8+ T cells was reduced compared with control CD8+ T cells (range observed in three independent experiments was: 0.2–0.7 ng/ml IL-5 in WAS CD8+ T cells; 3.2–22.6 ng/ml IL-5 in control CD8+ T cells) (data not shown). IL-4 and IL-10 were low or undetectable (0–400 pg/ml) in the supernatants of both WAS and control CD8+ T cells (data not shown).

In accordance with the results of cytokine secretion, a strong reduction in the proportion of cells producing IL-2 and IFN-γ in response to TCR and CD28 triggering was observed by intracellular staining in all WAS patients (Fig. 4B) (range of IL-2+ cells in HDs, n = 5, was 29–47%; range of IFN-γ+ cells was 26–65%). Similarly to what was shown for CD4+ T cells, CD8+ T cells from the XLT patient displayed normal production of IL-2 and suboptimal IFN-γ production. In different experiments, levels of IL-2 and IFN-γ production by CD8+ T cells from this patient were either within or below normal range, and consistently higher than those found in WAS CD8+ T cells. Although stimulation with TPA/ionomycin induced robust production of IL-2 and IFN-γ by CD8+ T cells from all patients (Fig. 4B), in patients W2, W8, and W12, a reduction in cells producing both IL-2 and IFN-γ was still present (range of IL-2+/IFN-γ- cells in HDs, n = 5, was 75–89%). Despite defective cytokine production, CD8+ T cells from patient W1 were found to be able to lyse both allogenic EBV-transformed B cells and allogenic PHA blasts. When EBV-B cells were used as targets, the average percentages of specific lysis were as follows: W1 54 ± 7.1%; HDs 47.3 ± 11.9% (E:T ratio 90:1); W1 37.5 ± 0.7%; HDs 31.8 ± 16.1% (E:T ratio 30:1).

Together, these results indicate a profound defect of IL-2, IFN-γ, and TNF-α secretion in CD8+ T cells from WAS patients, which, for IL-2 and IFN-γ, corresponds to reduced protein synthesis.

WASP is required for IL-2 and IFN-γ gene transcription by CD4+ and CD8+ T cells

To test whether a defect in transcription could be responsible for the impairment in IL-2 and IFN-γ production, cytokine mRNA levels were analyzed by RPA on CD4+ (Fig. 5A) and CD8+ (Fig. 5B) T cell lines from patients W1 and W2 and from two HDs. Quantification of one representative experiment shows that upon TCR/CD28-mediated stimulation, levels of both IL-2 and IFN-γ mRNA were lower in WAS CD4+ T cells as compared with control CD4+ T cells (Fig. 5C). This reduction was observed in three independent experiments (W1: IL-2 mRNA = 10–52% of the normal values; W2: IL-2 mRNA = 22–63% of the normal values). In contrast, IL-4 mRNA was...
produced by WAS CD4⁺ T cells at higher levels than those observed in control cells, while production of IL-10 mRNA was in the normal range (Fig. 5C). Independent of the absolute values, CD4⁺ T cells from WAS patients stimulated through TCR/CD28 reproducibly showed higher IL-4 vs IFN-γ mRNA ratio than control CD4⁺ T cells, indicating a difference in the balance between these two cytokines. Similarly, following anti-CD3/CD28 mAb stimulation, WAS CD8⁺ T cells produced low levels of IL-2 and IFN-γ mRNA, as compared with control CD8⁺ T cells (Fig. 5D). TPA plus ionomycin stimulation induced comparable IL-2 and IFN-γ mRNA levels in both WAS and control CD8⁺ T cells and CD4⁺ T cells. These findings indicate that defective cytokine production in WAS T cells is dependent on TCR/CD28-mediated stimulation and is determined at the transcriptional level.

Reduced T-bet mRNA induction in WAS CD4⁺ T cells stimulated via TCR and CD28

We next analyzed the levels of T-bet and GATA-3 mRNA in WAS CD4⁺ T cell lines, as these transcription factors are involved in the transcription of IFN-γ and IL-4 genes, respectively (37, 38). Levels of both T-bet and GATA-3 mRNA were comparable in resting CD4⁺ T cells from W1 and W2 patients and healthy controls (Fig. 6). Upon stimulation with 1 μg/ml anti-CD3 plus 10 μg/ml anti-CD28 mAb, T-bet was induced in both control and WAS CD4⁺ T cells, but at lower levels in the latter (Fig. 6A, p < 0.05 for patient W2 compared with HDs). At a suboptimal dose of anti-CD3 (0.1 μg/ml) plus anti-CD28 mAb, T-bet mRNA expression was significantly higher in control cells than in WAS CD4⁺ T cells (Fig. 6A, p < 0.05 for both W1 and W2 patients). Stimulation with TPA plus ionomycin induced comparable T-bet expression in WAS and control CD4⁺ T cells (data not shown). At high doses of anti-CD3 mAb (1 μg/ml), GATA-3 mRNA was expressed by WAS and control cells at comparable levels (Fig. 6B). At the lower anti-CD3 mAb dose (0.1 μg/ml), GATA-3 mRNA was not induced in WAS CD4⁺ T cells whereas only a fraction of control T cells expressed it (Fig. 6B). These data show that CD4⁺ T cells from WAS patients have a reduced ability to induce T-bet mRNA upon TCR/CD28-mediated stimulation.

Defective NFAT nuclear levels in CD4⁺ and in CD8⁺ WAS T cells

Defective NFAT-1 activation and Fos expression have been recently shown to be associated to defective IL-2 gene transcription in T cells from WASP knockout mice (31). Therefore, we investigated the nuclear levels of NFAT family members (NFAT-1 and NFAT-2) and Fos family members in T cell lines from WAS patients. Recruitment of the dephosphorylated forms of NFAT-1 and NFAT-2 to the nucleus was observed in both WAS and control CD4⁺ T cells, as early as 10 min after stimulation through TCR/CD28 and persisted for at least 120 min (Fig. 7A). However, in WAS CD4⁺ T cells, the levels of nuclear NFAT-1 were lower than in control CD4⁺ T cells at the 10-min time point (Fig. 7B). This difference was observed in three of four independent experiments. At later time points, comparable levels of nuclear NFAT-1 were detected in WAS and control CD4⁺ T cells. Nuclear recruitment of NFAT-2 was observed after a 10-min stimulation and persisted for at least 120 min, at comparable levels in WAS and control CD4⁺ T cells (Fig. 7, A and B). WAS and control CD4⁺ T cells showed a similar profile of expression of Fos family members, which were induced at 30 min and further increased 120 min after stimulation (Fig. 7, A and B). In WAS CD8⁺ T cells, a significant reduction in the nuclear levels of both NFAT-1 and NFAT-2 was observed 10 min after TCR/CD28 triggering (Fig. 7, C and D; NFAT-1 p < 0.05; NFAT-2 p < 0.01). Although in WAS CD8⁺ T cells, reduced levels of NFAT-1 and NFAT-2 were also found at later time points (Fig. 7, C and D), this reduction was not significant. Fos family members were expressed at similar levels in WAS and control CD8⁺ T cells 120 min after TCR/CD28 triggering (Fig. 7, C and D). These data indicate that the absence of WASP in human CD4⁺ and CD8⁺ T cells, stimulated through TCR/CD28, leads to a reduction in the early nuclear recruitment of NFAT proteins, while the expression of Fos proteins appears normal.

Discussion

In the present study, we investigated the function of purified CD4⁺ and CD8⁺ T cells from WAS patients. To define their respective role in the mechanisms underlying WAS-associated immunodeficiency and to gain novel insights into the contribution of WASP in the activation and effector functions of T cells, the cytokine production profile of untransformed WASP-deficient T cell lines was analyzed. Previous studies in T cell lines from WAS patients suggested that WASP deficiency induces a general state of unresponsiveness upon TCR-driven stimulation (4, 25). Here, we demonstrate that WASP is specifically required for the production of Th1 cytokines by CD4⁺ and CD8⁺ T cells and for the regulation of the transcriptional activation of Th1 cytokine genes.

CD8⁺ T cells from WAS patients show a strong defect in the production of IL-2, IFN-γ, and TNF-α which is associated to defective TCR/CD28-mediated proliferation. These data are in accordance with data obtained in vivo. Indeed, reduced frequency of Ag-specific CD8⁺ T cells and defective cytokine production have been previously reported in WAS knockout
mice, in which the clearance of influenza A virus upon primary infection was normal, but the secondary response was impaired (29, 39). In response to TCR/CD28-driven stimulation, WAS CD4⁺ T cells display impaired production of Th1 cytokines (IL-2, IFN-γ, and TNF-α), while the production of Th2 cytokines (IL-4, IL-10, and IL-5) is minimally affected. Importantly, this Th1 cytokine defect was confirmed in fresh WAS T cells, because PBMC from two WAS patients displayed impaired IL-2 and IFN-γ secretion upon TCR/CD28 stimulation (data not shown). These results are not due to an intrinsic inability of WASP-deficient T cell lines to produce Th1 cytokines, as stimulation with TPA plus ionomycin induces high levels of both Th1 and Th2 cytokines in these cells. Moreover, T-bet and GATA-3 mRNA are comparable in resting WAS and controls CD4⁺ T cells. Therefore, defective production of Th1 cytokines reflects a defect in TCR/CD28-mediated signaling due to the absence of WASP. The impairment in IL-2 and IFN-γ production is present at both intracellular and extracellular protein levels, thus excluding a secretion defect. In addition, we show that the pattern of cytokine production by WAS T cells is consistent with the pattern of cytokine gene expression. Indeed, reduced levels of IL-2 and IFN-γ mRNA are found in WAS CD4⁺ T cells stimulated through TCR/CD28 and analyzed at different time points (data not shown). Previous results in T cells from WASP knockout mice showed that WASP is required for IFN-γ secretion from intracellular stores but not for its synthesis (28). These different results could be ascribed either to differences between human WAS cells and murine WASP knockout cells, or to different culture conditions. Interestingly, after stimulation with anti-CD3/CD28 mAb, IL-4 mRNA levels are higher in WAS CD4⁺ T cells than in control cells. Because this difference is not found at the protein level, we cannot
excludes a posttranscriptional defect in IL-4 production in WAS CD4\(^+\) T cells. An important finding is that the balance between IFN-\(\gamma\) and IL-4 mRNA is different between WAS patients and control CD4\(^+\) T cells. Indeed, in response to TCR/CD28-mediated stimulation, control cells reproducibly show higher levels of IFN-\(\gamma\) than IL-4 mRNA, while WAS CD4\(^+\) T cells show comparable levels of the two mRNA species. Our findings that T-bet is induced at lower levels in WAS than in control CD4\(^+\) T cells (especially at low anti-CD3 mAb dose) supports a role of WASP in regulating T-bet expression and, in turn, IFN-\(\gamma\) gene transcription. Interestingly, the T-bet defect is less pronounced upon stimulation with higher doses of anti-CD3 mAb (especially in W1 CD4\(^+\) T cells) and is not present upon TPA plus ionomycin stimulation (data not shown), suggesting that the higher threshold required for activation of WASP-deficient CD4\(^+\) T cells (4, 30) could contribute to the IFN-\(\gamma\) defect. T-bet has been clearly shown to be required for IFN-\(\gamma\) production and Th1 differentiation by CD4\(^+\) T cells, whereas it seems dispensable for IFN-\(\gamma\) production by CD8\(^+\) T cells (37). Cooperation between T-bet and eomesodermin, a T-bet paralogue, may be more relevant for the acquisition of effector functions by CD8\(^+\) T cells (40).

New insights into the role of WASP in TCR/CD28-mediated signal transduction have been recently provided by a study performed in T cells of WASP knockout mice, showing defective NFAT-1 activity (and expression of Fos mRNA) in association with defective IL-2 gene transcription (31). In CD4\(^+\) T cells from WAS patients, a reduction in the nuclear levels of NFAT-1, but not NFAT-2, is observed early after TCR/CD28 triggering. This is in accordance with the normal production of Th2 cytokines by WAS CD4\(^+\) T cells, because NFAT-2 has been shown to be necessary mainly for transcription of Th2 cytokine genes in CD4\(^+\) T cells (41). In contrast, it has been shown that T-bet positively regulates the binding of NFAT-1 to the IFN-\(\gamma\) promoter (42). Overall, our data suggest that NFAT-1 and T-bet defects contribute to the selective defect in Th1 cytokine production by WAS CD4\(^+\) T cells. Furthermore, in WAS CD8\(^+\) T cells, reduced nuclear levels of both NFAT-1 and NFAT-2 could in part account for the defects in cytokine gene transcription. In agreement with our findings, a defect in early NFAT-2 nuclear translocation has been recently reported in NK cells from WAS patients (32). In T cells from WASP knockout mice, defective NFAT-1 activity has been found to be associated with reduced Fos induction. However, our data on human WAS CD4\(^+\) and CD8\(^+\) T cells fail to show any significant alteration in Fos expression. This discrepancy could be due to the difference between the two models. The defects in the activation of multiple transcription factors identified here in human WAS T cells, and in previous studies in different cellular models (13, 30–32), suggest that rather than controlling a single signaling pathway downstream of the TCR, WASP may regulate the threshold for activation of different pathways converging toward the transcriptional induction of cytokine genes.

Our data indicate that WASP deficiency severely impairs the production of Th1 cytokines. However, NFAT and T-bet pathways, although clearly reduced by WASP deficiency, are not blocked. Therefore, other mechanisms involved in the regulation of Th1 cytokine production could be altered in WAS T cells. WASP deficiency has been shown to impair immunological synapse formation as well as actin cytoskeleton reorganization (4, 5), two crucial events required for optimal T cell activation. Therefore, cytoskeletal and signaling alterations could contribute to the Th1 transcriptional defect observed in WAS T cells. Moreover, it has been proposed that WASP could intervene in TCR signaling in T cells, as well as in NKp46 signaling in NK cells, independently on its role in actin polymerization and immunological synapse formation (30, 32, 43). It has been recently demonstrated that the nuclear translocation of the WASP homolog N-WASP regulates gene transcription in nonhemopoietic cells (44). Therefore, it could be hypothesized that a similar mechanism contributes to the regulation of cytokine gene transcription by WASP in T cells.

We and others previously showed that the inability of WASP-deficient mature human T cells to proliferate and to produce IL-2, in response to TCR stimulation, is a direct consequence of WASP absence, because these defects could be fully corrected by WAS gene transfer (45, 46). In addition, upon TCR stimulation, WAS gene transfer was able to restore normal expression of GM1, a ganglioside enriched in lipid rafts. However, WASP is already expressed in human CD34\(^+\) progenitor cells (33) and it could therefore play a role not only in the activation of mature T cells, but also in T cell maturation. WAS patients have reduced numbers of circulating naive T cells, which could be due either to reduced thymic output or to reduced proliferation/survival of mature T cells (22). The finding of a normal pattern of TCR \(\beta\) usage in T cells from young WAS patients supports that T cell development is relatively independent of WASP expression (47). In accordance, data obtained from WASP knockout mice indicate that WASP deficiency does not significantly impair T cell maturation (26, 27, 48). However, it cannot be ruled out that some of the defects reported in this study could derive from intrinsic T cell abnormalities acquired during development in the absence of WASP.

Because it has been shown that naive CD4\(^+\) T cells stimulated with low doses of Ag preferentially differentiate into IL-4- rather than IFN-\(\gamma\)-producing cells (49), it is possible that in WAS patients, priming of T cells results in weak T cell activation, with the generation of memory cells which expand poorly and preferentially produce IL-4, rather than IFN-\(\gamma\), upon Ag rechallenge. This hypothesis is consistent with the clinical phenotype of WAS patients, who often suffer from infections by intracellular pathogens and have high levels of circulating IgE and eczema (19). Interestingly, naive WAS CD4\(^+\) T cells can be polarized toward a Th1 phenotype, as indicated by increase in IFN-\(\gamma\) production and concomitant repression of IL-4 production upon TPA/ionomycin-mediated stimulation (data not shown). However, the frequency of cells producing both IL-2 and IFN-\(\gamma\) upon TCR/CD28 stimulation remains strongly defective. We can hypothesize that, in WAS patients, IL-2 and IFN-\(\gamma\) deficiency leads to a severe impairment in the capacity to mount protective Th1 responses. Moreover, defective IL-2 and IFN-\(\gamma\) production by CD4\(^+\) T cells could result in a reduced help toward CD8\(^+\) T cells during viral infections. The generation of CD8\(^+\) T cell memory and, in particular, the ability of CD8\(^+\) T cells to expand in response to secondary stimulation requires CD4\(^+\) T cell help at the time of priming (50). Therefore, our data support the hypothesis that CD8\(^+\) T cells from WAS patients suffer from both an intrinsic defect and inadequate help by Th1 cells. Interestingly, we found that WAS CD8\(^+\) T cells can lyse normally both allogenic PHA blasts and allogenic EBV-transformed B cells. It is possible that, in WAS patients, the inability to clear viral infections is not due to a defective lytic ability, but rather to the inability of Ag-specific CD8\(^+\) T cells to expand upon Ag exposure. Moreover, it is possible that defective IFN-\(\gamma\) production, together with a reported defect of NK activity (51), is
responsible for the increased susceptibility not only to viral infections, but also to lack of immune surveillance leading to hemolytic malignancies. In conclusion, our data contribute to a better understanding of the role of WASP in regulating CD4 T and CD8 T cell functions and of the immunological mechanisms underlying WASP and its characteristic clinical manifestations. In addition, in the context of the development of a gene therapy protocol for WAS patients (29, 45, 46, 52–55), our study better defines the immunological defects that will need to be assessed and corrected. Finally, the newly identified role of WASP in driving Th1 cytokine production indicates that modulation of WASP activity could in principle be envisaged as a strategy for treatment of Th1-mediated pathologies such as Crohn’s disease, psoriasis, and multiple sclerosis (56–58).

Acknowledgments

We thank Anna Mondino, Anna Stella, Stefan Feske, and Doreen Cantrell for helpful discussions. We also thank Anjana Rao and Stefan Feske for the anti-NFAT-1Ab. We are grateful to the patients and their families.

Disclosures

The authors have no financial conflict of interest.

References


