Systemic autoimmunity and defective Fas ligand secretion in the absence of the Wiskott-Aldrich syndrome protein

*Nikolay P. Nikolov,1,2 *Masaki Shimizu,3 *Sophia Cleland,2 Daniel Bailey,2 Joseph Aoki,4 Ted Strom,5 Pamela L. Schwartzberg,4 Fabio Candotti,3 and Richard M. Siegel2

1Office of the Clinical Director and 2Immunoregulation Group, Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), National Institutes of Health (NIH), Bethesda, MD; 3Disorders of Immunity Section, Genetics and Molecular Biology Branch (GMBB) and 4Cell Signaling Section, Genetic Disease Research Branch, National Human Genome Research Institute (NHGRI), NIH, Bethesda, MD; and 5Pathology and Laboratory Medicine Service, Memphis VA Medical Center and Department of Pathology, University of Tennessee Health Science, Memphis

Autoimmunity is a surprisingly common complication of primary immunodeficiencies, yet the molecular mechanisms underlying this clinical observation are not well understood. One widely known example is provided by Wiskott-Aldrich syndrome (WAS), an X-linked primary immunodeficiency disorder caused by mutations in the gene encoding the WAS protein (WASp) with a high incidence of autoimmunity in affected patients. WASp deficiency affects T-cell antigen receptor (TCR) signaling and T-cell cytokine production, but its role in TCR-induced apoptosis, one of the mechanisms of peripheral immunologic tolerance, has not been investigated. We find that WASp-deficient mice produce autoantibodies and develop proliferative glomerulonephritis with immune complex deposition as they age. We also find that CD4+ T lymphocytes from WASp-deficient mice undergo reduced apoptosis after restimulation through the TCR. While Fas-induced cell death is normal, WASp deficiency affects TCR-induced secretion of Fas ligand (FasL) and other components of secretory granules by CD4+ T cells. These results describe a novel role of WASp in regulating TCR-induced apoptosis and FasL secretion and suggest that WASp-deficient mice provide a good model for the study of autoimmune manifestations of WAS and the development of more specific therapies for these complications. (Blood. 2010;116(5):740-747)

Introduction

Wiskott-Aldrich Syndrome (WAS) is an X-linked primary immunodeficiency that affects the development and function of multiple hematopoietic cell lineages, including, T, B, and natural killer (NK) cells, dendritic cells, and platelets.1,2 Clinically, WAS is characterized by the clinical triad of thrombocytopenia, eczema, and susceptibility to infection. Autoimmune complications are also surprisingly common in WAS, occurring in 40% to 70% of patients in retrospective cohort studies, and are becoming increasingly frequent clinical management issues as these patients live longer due to more effective prophylaxis and treatment of infectious complications.3-5 Frequent autoimmune disorders in WAS patients can include autoimmune hemolytic anemia, thrombocytopenia, nephritis, vasculitis, and inflammatory bowel disease.3-4 Interestingly, autoimmune disorders are also common complications in posthematopoietic cell transplant WAS patients when mixed chimerism is obtained.5

Most cases of WAS can be linked to loss of function mutations in the WAS protein (WASp).6-8 WASp is a multidomain 502 amino acid cytoplasmic protein expressed specifically in hematopoietic cells.6 In T cells, WASp is activated by the T-cell antigen receptor (TCR) through the small G protein Cdc42 or tyrosine phosphorylation. WASp induces branched-actin polymerization through interactions with the ARP2/3 complex. WASp deficiency results in defective formation of the immunologic synapse in WASp-deficient T cells and NK cells.6,9-12 WASp-deficient T cells proliferate poorly after T-cell receptor stimulation, but this can be largely rescued by addition of exogenous interleukin-2.13

We and others have found a defect in regulatory T-cell (Treg) homeostasis and function in WAS deficiency,14-17 providing one possible mechanism that could predispose WAS patients to develop autoimmunity. Whether TCR-induced cell death, another mechanism of peripheral immune tolerance, is affected by WASp deficiency has not been investigated. Activated T cells can undergo apoptosis in response to stimulation through the TCR, a process termed restimulation-induced cell death (RICD).18-20 This pathway can eliminate T cells responding to chronically expressed antigens, such as autoantigens and pathogens in persistent infections.21-23 In CD4+ T cells, much of this RICD depends on autocrine interactions of the tumor necrosis factor (TNF) family member Fas ligand (FasL) and its receptor Fas/CD95.20,24 Fas or FasL deficiency results in systemic autoimmunity in humans and mice,25,26 and more recently it has been found that deficiency of Fas in T cells, B cells, or dendritic cell lineages can independently lead to autoantibody production in animal models.27,28 Given the T-cell signaling defects described in WASp-deficient T lymphocytes, we hypothesized that in addition to affecting aspects of T-cell activation, WASp deficiency may impair the TCR-induced RICD pathway, contributing to the breakdown of self-tolerance and autoimmunity. Indeed, we show here that T cells from Was knockout (KO) mice have defective production of biologically active FasL after restimulation through the TCR. These defects may contribute to the development of age-dependent production of autoantibodies and immune-complex nephritis that we have seen in these animals and play a role in the onset of autoimmunity disease in patients with WAS.


* N.P.N., M.S., and S.C. contributed equally to this work.

The online version of this article contains a data supplement.
Methods

Animals

Wasp-deficient mice on the 129 background (129S6/SvEvTac-Huqtm1Bby/J) were obtained from The Jackson Laboratory. Some mice were backcrossed for 3 generations to C3H/HcJ and for 8 generations to C57Bl/6J, and sera were sampled as described. Mice were maintained in SPF conditions, and experiments were carried out according to the Animal Care guidelines of the National Institutes of Health (NIH) Intramural Research Program (IRP) and the Memphis VA Medical Center. All mouse experiments were approved by the NIH IRP.

Measurement of autoantibodies and circulating immune complexes

The presence of antinuclear antibodies (ANA) was determined by immunofluorescence staining of fixed HEp-2 cells (Antibodies). Cells were incubated with 1:40 diluted sera and then with Alexa 488–conjugated goat anti–mouse immunoglobulin G (IgG) antiserum (Invitrogen). Fluorescence was evaluated using fluorescent microscopy by 3 blinded observers (N.P.N., D.B., and R.M.S.) with very good interobserver reproducibility. Positive staining was defined as a distinct staining within the nucleus brighter than the staining evident in the cytoplasm. Positive sera were further serially diluted until they became negative for nuclear immunofluorescence. ANA-positive sera were tested for anti-double–stranded DNA (anti-dsDNA) using mouse enzyme-linked immunosorbent assay (ELISA; Alpha Diagnostic International), following the manufacturer’s instructions. The cutoff for a positive anti-dsDNA was 2 times the absorbance of the negative control corrected for blank values. Serum circulating immune complexes were measured by ELISA according to the manufacturer’s protocol (Alpha Diagnostic International).

Kidney immunofluorescence and measurement of proteinuria

Sections of one of the kidneys were fixed in buffered formalin. Five-micron sections were stained with periodic acid–Schiff (PAS) and were processed to reveal Congo red deposits. Sections of one of the kidneys were fixed in buffered formalin. Five-micron sections were stained with periodic acid–Schiff (PAS) and were processed to reveal Congo red deposits. Kidney immunofluorescence and measurement of proteinuria were sampled as described. Mice were maintained in SPF conditions, and sera were obtained from The Jackson Laboratory. Some mice were backcrossed for 3 generations to C3H/HeJ and for 8 generations to C57Bl/6J, and sera were sampled as described. Mice were maintained in SPF conditions, and experiments were carried out according to the Animal Care guidelines of the National Institutes of Health (NIH) Intramural Research Program (IRP) and the Memphis VA Medical Center. All mouse experiments were approved by the NIH IRP.

Measurement of fasL mRNA, protein, and function

RNA was prepared using Trizol (Invitrogen) and RNeasy mini kit (Qiagen) and amplified using one-step Superscript II RT-PCR (Invitrogen). Applied Biosystems primer/probe sets for fasL and β-2 microglobulin as standard were used. RNA induction was quantified using the ΔCt method. To evaluate fasL secretion, supernatants from the cell death assays were collected at 6 hours, and the amount of FasL was quantified using mouse ELISA (Fas Ligand/TNFFSF6 Duoset; R&D Systems) according to the manufacturer’s recommendations. FasL surface levels were tested by FACS using anti-CD178-PE (BD Pharmingen). FasL was collected from activated T cells cultured at 4 × 10⁶ cells/mL with anti-CD3 for 6 hours in the presence of IL-2. Supernatants were prespun at 10 000 g for 30 minutes to remove cellular debris, and cell-free supernatant was then fractionated by centrifugation through Microcon YM-100 membranes after preswelling with a 1% bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS). The concentrated fraction (≥ 100 kDa) was rediluted to the original volume. FasL activity was assayed on WEHI-279 B cell lymphoma cells (ATCC) at 2.5 × 10⁶ cells/mL mixed at a 1:3 ratio with T-cell supernatants for 23 hours. Apoptosis was measured with the Cell Titer Glo cell viability assay (Promega). Anti-FasL (10 μg/mL, MFL3; BD Pharmingen) in the presence of Fc receptor blockade with 2.4G2 was added to some samples to determine whether apoptosis was caused by FasL. Purified soluble FasL was obtained from R&D Systems, and vesicular FasL was obtained from Uptake/Millipore.

β-Hexosaminidase release assay

Degranulation induced via TCR restimulation was determined by β-hexosaminidase release. CD4⁺ or CD8⁺ T cells were incubated at 5.0 × 10⁵ cells/mL in Tyrode buffer (135mM NaCl, 5mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, 5.6mM glucose, 20mM HEPES, pH 7.4). Cells were then restimulated with plate-bound murine anti-CD3ε in the presence of 50 U/mL IL-2 for 4 hours at 37°C. The reaction was terminated by centrifugation at 4°C. The supernatant was collected, and the total hexosaminidase concentration was determined by cell lysis in 1% Triton X-100. Aliquots of the supernatants and total cell lysates were incubated with 50μL 1mM p-nitrophenyl-N-acetyl-D-glucopyranoside (p-NAG; Sigma-Aldrich) substrate for 1 hour at 37°C in 0.1 M sodium citrate buffer (pH 4.5) at 37°C. The reaction was terminated by the addition of 100 μL 0.1 M carbonate/bicarbonate buffer. The release of the product 4-p-nitrophenol was read by optical absorbance at 405 nm.

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Percentage of β-hexosaminidase release was calculated as follows: (stimulated sup – unstimulated sup/unstimulated total) × 100.

**Statistical analysis**

Quantitative statistics were computed by Student t test. Categorical variables were assessed using Fisher exact and χ² tests. Two-sided P values were used for all analyses, and the level of statistical significance was set a priori at .05. Statistical analyses were performed using SAS E-Guide, Version 3 for Windows (SAS Institute) and Prism Version 4 for Macintosh (GraphPad Software).

**Results**

To screen for manifestations of autoimmunity in a genetically defined animal model of WAS, we first evaluated autoantibody production in WASp-deficient mice. Sera from WASp-deficient mice on a 129SvEvTac background were screened for ANA and WASp contributes to RICD, we examined the ability of activated T cells to undergo apoptosis in response to TCR stimulation (Figure 3A). Purified CD4+ T cells from WASp-deficient mice were activated for 2 days and expanded in the presence of IL-2 for 3 days. Then RICD was induced by restimulation with plate-bound anti-CD3. Cell death under these conditions was dependent on...
FasL-Fas interactions, as blocking antibodies against FasL completely abrogated cell death (data not shown). TCR-induced cell death was significantly reduced in WASp-deficient T cells over a wide range of concentrations of anti-CD3. This was not due to differences in the surface expression of Fas or the TCR, because FACS staining revealed similar levels of these molecules on the surface of activated T cells from WASp-deficient and control mice (Figure 3B). TCR-induced apoptosis requires cell cycle progression through G1/S phase, so reduced apoptosis of WASp-deficient T cells could be due to reduced cycling. However, thymidine incorporation by WASp-deficient cells was similar to controls after 3 days of activation (Figure 3C). Cell division in WASp-deficient cells as assayed by CFSE dilution was initially delayed at 48 hours after initial activation, but was then comparable to controls during the expansion phase of culture when RICD is measured (supplemental Figure 2). Apoptosis induced by addition of a biologically active form of oligomerized FasL was intact (Figure 3D), indicating that the reduced TCR-induced cell death of WASp-deficient T cells is not due to a failure of Fas signaling. Previous studies have noted accelerated spontaneous apoptosis of cells from WAS patient lymphocytes. This may be ascribed to enhanced apoptosis in the absence of survival promoting cytokines. To determine the sensitivity of WASp-deficient T cells to this type of cell death, we cultured activated CD4+ T cells in the absence of cytokines and monitored viable cell number and viability (Figure 3E-F). Cell numbers were slightly lower, but viability was comparable between WASp and control T cells, with both showing significant cell death after withdrawal of IL-2. Thus, changes in the intrinsic cell death pathway do not appear to compensate for defective TCR-induced death of WASp-deficient T cells.

Because FasL-induced apoptosis was normal in WASp-deficient cells, WASp may instead regulate FasL production or secretion after TCR stimulation. We measured FasL mRNA induction in response to TCR stimulation of activated CD4+ T cells from WASp-deficient mice and controls. As shown in Figure 4A, induction of FasL mRNA was not significantly different between T cells from WASp-deficient mice and controls. FasL was also normally up-regulated on the plasma membrane after TCR restimulation in WASp-deficient T cells (Figure 4B). These results indicate that the components of TCR signaling responsible for the transcriptional induction of FasL and transport to the plasma membrane are not dependent on WASp.

In addition to expression on the plasma membrane as a type II transmembrane protein, FasL can also be secreted as a soluble molecule or in secretory vesicles. Soluble FasL is produced after cleavage of the extracellular domain by metalloproteinases, and this form of FasL has generally been found to be nonfunctional in inducing cell death. FasL is secreted in membrane-bound form on the surface of exosomal vesicles derived from secretory lysosomes that contain enzymes such as granzymes and β-hexosaminidase. This form of FasL has been shown to be potently cytotoxic for Fas-expressing target cells. To test whether WASp might affect FasL secretion through either of these pathways, we measured FasL present in the supernatant of restimulated T cells from WASp-deficient and control T cells (Figure 5A). We found reduced amounts of FasL in the supernatant of restimulated WASp-deficient T cells. To determine whether WASp is required for the secretion of FasL in exosomes or in soluble form, we filtered the supernatants of restimulated WASp-deficient or control T cells through 100-kDa cutoff membranes that concentrate FasL in high-molecular weight vesicles but allow flow through of soluble FasL (supplemental Figure 3). WASp-deficient restimulated T cells secreted less FasL in both of these fractions (Figure 5B). To determine whether the reduced FasL secretion was functionally significant, we added T-cell supernatants to the Fas-sensitive WEHI-279 lymphoma cell line. As previously described, purified vesicular, but not soluble, FasL induced cell death in these cells, and a FasL blocking antibody could inhibit the cell death induced by vesicular FasL (Figure 5C). We then applied the supernatants from restimulated WT or WASp-deficient T cells to WEHI-279 cells to measure the cytotoxicity of these fractions (Figure 5D). Significant cytotoxicity could be found in the high-molecular weight fraction of WT T-cell supernatants and this could be blocked by anti-FasL, indicating that FasL was the principal cytotoxic molecule present in this fraction. The cytotoxicity of the high-molecular weight fraction of supernatants from WASp-deficient T cells was significantly reduced compared with WT.
supernatants in this assay. The residual cytotoxicity in the supernatant of WASp-deficient cells was partially inhibited by FasL blockade, but not as strongly as in WT supernatants. The low molecular weight fraction of both WT and WASp-deficient T cells induced less than 10% cytotoxicity (data not shown). To determine whether WASp more generally affects release of secretory lysosomes, we measured specific release of /H252-hexosaminidase by WASp-deficient and control CD4 and CD8 T cells after TCR restimulation. Secretion of /H252-hexosaminidase was significantly reduced in both CD4 and CD8 activated, WASp-deficient T cells (Figure 5E), suggesting that the defect in FasL secretion was tied to reduced secretion of exosomes by restimulated WASp-deficient T cells. Taken together, this data suggests that the decreased cell death in restimulated WASp-deficient T cells results at least in part from a deficiency in the production of biologically active FasL.

Discussion

We describe here a high incidence of autoantibodies and immune-complex nephritis in WASp-deficient mice. The pathologic features or renal impairment in WASp-deficient mice are not as severe as seen in complete Fas deficiency on a Murphy Roths Large (MRL) background or the NZB/NZW mouse model, but are reminiscent of human IgA nephropathy that has been reported in WAS. Anti-dsDNA antibodies were also increased in the serum of ANA-positive WASp-deficient mice. Since we see apoptosis defects in mice less than 3 months of age, before the development of autoantibodies, and autoantibodies are not seen in every animal, additional environmental factors likely play a role in the production of autoantibodies in the setting of WASp deficiency. Other mechanisms, such as defects in regulatory T cells in WAS deficiency may also contribute to the development of autoimmunity. Nevertheless, our findings suggest
that WASp-deficient mice represent a useful model of the autoimmune and renal manifestations in WAS.

These results show for the first time that WASp is important not only in antigen-driven primary T-cell activation, but also in TCR-mediated restimulation and apoptotic cell death of previously activated T cells. Unlike the situation in lpr mice or the autoimmune lymphoproliferative syndrome, Fas-induced apoptosis is intact in WASp-deficient T cells, and the defect in FasL function is not as severe as is seen in FasL mutant gld mice.26 For this reason, it is not surprising that WASp-deficient mice do not show all the features of Fas or FasL deficiency, such as generalized lymphadenopathy and accumulation of peripheral CD4+CD8+ “double-negative” T cells. We have looked for but not observed anti-red blood cell (RBC) antibodies that are seen in WAS patients nor the colitis that has been observed by others in WASp-deficient mice. However, it is interesting that the colitis associated with WASp deficiency has recently been shown to be transferable by T cells.39 We are currently performing experiments to specifically re-express WASp in T cells of WASp-deficient mice to determine whether the autoantibody production we see in WASp deficient mice stems from a T-cell intrinsic defect.

Our data suggests that reduced secretion of biologically active FasL contributes to inefficient TCR-induced apoptosis in the absence of WASp. Soluble FasL was also reduced in the supernatants of WASp-deficient T cells, suggesting that metalloprotease-mediated cleavage of FasL may also be reduced in WASp deficiency, although this did not result in measurable increases in surface FasL. Reduced apoptosis of WASp-deficient T cells is more likely attributable to the deficiency in membrane-bound FasL, since only this form of FasL induces significant cytotoxicity and protects against autoimmunity.35 The fact that surface expression of FasL was normal despite reduced RICD suggests that the vesicular form of FasL may be important in autocrine T-cell death.

WASp may influence trafficking of FasL into secretory lysosomes or may more generally affect trafficking of secretory lysosomes into multivesicular bodies and fusion of these structures with the plasma membrane.36,40 The reduced release of the secretory lysosome component β-hexosaminidase by WASp-deficient T cells supports the notion of a more generalized granule secretion defect in WASp deficiency. WASp-deficient T cells have been found to have defects in secretion of other cytokines such as IL-2 and interferon-γ (IFN-γ).41 Whether or not these defects result from the same mechanism that impairs vesicular FasL secretion is not known. Other cell types such as mast cells and NK cells have been shown to have defects likely related to abnormal granule secretion in the setting of WASp deficiency.42,43 Although B cell–specific deletion of Fas can result in autoantibody production,43 the mechanism we have uncovered here of defective FasL production is unlikely to affect B cells directly, as B cells are generally not thought to produce Fas ligand in sufficient quantity to induce autocrine apoptosis within the B cell compartment. Interestingly, other immunodeficiencies with reduced granule exocytosis, such as Griscelli syndrome associated with Rab27a deficiency and Hermansky-Pudlak syndrome due to AP3 deficiency, predispose patients to immunopathologic complications such as the hemophagocytic syndrome. Whether secretion of FasL is defective in these diseases is also not known.

Other mechanisms may also contribute to the pathogenesis of autoimmunity in WASp deficiency. Delayed phagocytosis of apoptotic cells has been reported in WASp deficiency and might be another potential mechanism of loss of peripheral tolerance.45

Figure 5. Reduced bioactive FasL and granule secretion by WASp-deficient T cells. (A) Secreted FasL measured by ELISA in supernatants after 6 hours of stimulation of activated CD4+ T lymphocytes with the indicated concentrations anti-CD3 antibody. Values are the average ± SEM of data from 2 mice per group, and similar results were obtained in 3 independent experiments. (B) Supernatants from CD4+ T cells restimulated for 6 hour with anti-CD3 were fractionated by centrifugation through 100-kDa cutoff membranes, and FasL was quantitated in each fraction by ELISA. No FasL was detected in the < 100-kDa fraction when purified vesicular FasL was filtered through identical membranes. (C) The indicated concentrations of purified vesicular and soluble FasL were added to WEHI-279 cells, and cytotoxicity was quantitated by a luminescent cell viability assay. Anti-FasL antibody was added to demonstrate specificity of this assay for bioactive FasL. Specific cell death was quantitated as described in the methods. (D) FasL-dependent apoptosis-inducing activity of the indicated fractions of supernatants collected from cells WT and WASp-KO T cells. Supernatants from cells restimulated in C were assayed on WEHI-279 cells for apoptosis-inducing activity. Anti-FasL was added to the indicated samples to neutralize FasL activity. Asterisks mark the results of comparisons of WASp-KO with the identical WT cell supernatant fractions, and anti-FasL–treated samples compared with the same samples without anti-FasL. Results of P values from comparisons using Student unpaired t test are denoted as *P < .05, **P < .005, ***P < .001.

(E) β-Hexosaminidase release from activated WASp-deficient and control T cells restimulated with the indicated concentrations of anti-CD3 mAb. The curve of percent specific release was significantly different in WASp-deficient mice for both CD4 and CD8 than controls (P < .001, 2-way analysis of variance).
However, we have not found significant cell uptake defects in macrophages from WASp-deficient mice (supplemental Figure 4). The lymphopenia reported in WAS patients and WASp-deficient mice may also play an independent role in predisposing toward autoimmunity, although the degree of lymphopenia in WAS is not as severe as other human syndromes or mouse models associated with autoimmunity. The function and homeostasis of Treg are also affected by WASp deficiency. Since Treg may exert some of their suppressive effects through secretory lysosome components, WASp may regulate Treg function through similar mechanisms by which it controls TCR-induced cell death. Better understanding of these mechanisms may aid in the design of specialized therapies for autoimmune complications in WAS that avoid generalized immunosuppression, which is especially important in patients with concomitant immunodeficiency.

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Authorship

Contribution: N.P.N., M.S., D.B., S.C., and J.A. designed and carried out experiments and analyzed data; T.S. provided reagents and analyzed data; P.L.S., F.C., and R.M.S. interpreted data, designed experiments, and supervised the project; N.P.N., D.B., S.C., and R.M.S. wrote the paper; and all authors participated in editing the manuscript.

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The current affiliation for Nikolay P. Nikolov is Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research (NIDCR), NIH, Bethesda, MD. The current affiliation for Masaki Shimizu is Department of Pediatrics, School of Medicine, Institute of Medical, Pharmaceutical, and Health Sciences, Kanazawa University, Japan.

Correspondence: Richard M. Siegel, NIH, 10 Center Dr, Bldg 10, Rm 13C103, Bethesda, MD 20892, e-mail: siegelm@mail.nih.gov.

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