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Wiskott-Aldrich Syndrome Protein-Deficient Mice Reveal a Role for WASP in T but Not B Cell Activation

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Summary

The Wiskott-Aldrich syndrome (WAS) is a human X-linked immunodeficiency resulting from mutations in a gene (*WASP*) encoding a cytoplasmic protein implicated in regulating the actin cytoskeleton. To elucidate WASP function, we disrupted the *WASP* gene in mice by gene-targeted mutation. WASP-deficient mice showed apparently normal lymphocyte development, normal serum immunoglobulin levels, and the capacity to respond to both T-dependent and T-indepedent type II antigens. However, these mice did have decreased peripheral blood lymphocyte and platelet numbers and developed chronic colitis. Moreover, purified WASP-deficient T cells showed markedly impaired proliferation and antigen receptor cap formation in response to anti-CD3ε stimulation. Yet, purified WASP-deficient B cells showed normal responses to anti-Ig stimulation. We discuss the implications of our findings regarding WASP function in receptor signaling and cytoskeletal reorganization in T and B cells and compare the effects of WASP deficiency in mice and humans.

Introduction

The Wiskott-Aldrich syndrome (WAS) is characterized by severe immunodeficiency, thrombocytopenia, eczema, and lymphoreticular malignancies. Untreated patients die in the first decade of life (Rosen et al., 1995). Lymphocytes from WAS patients have an altered cytoskeleton with a reduction in cell surface microvilli as well as defects in antigen receptor–induced signaling (reviewed by Remold-O'Donnell et al., 1996). Linkage studies were employed to map the WAS gene to Xp11.22 (Kwan et al., 1991); the gene subsequently was isolated by positional cloning and found to encode a cytoplasmic protein (WASP) of 502 amino acids (Derry et al., 1994). Consistent with the cell types affected in WAS, the gene (*WASP*) was found to be expressed specifically in cells of the lymphocytic and megakaryocytic lineages (Derry et al., 1994).

WASP contains domains suggestive of a role in signaling and interactions with the cytoskeleton, including a pleckstrin homology domain, a GTPase-binding domain (GBD), a cofilin and verprolin homology domain found in other cytoskeletal-associated proteins, and a proline-rich C terminus that interacts with SH3-domain-containing molecules (Derry et al., 1994; Rivero-Lezcano et al., 1995; Banin et al., 1996; Bunnell et al., 1996; Cory et al., 1996; Finan et al., 1996; Miki et al., 1996; Symons et al., 1996). Although the majority of mutations in WAS patients are found in the N-terminal region of the protein, mutations have been found throughout the *WASP* gene, and most appear to eliminate or reduce expression of WASP (Derry et al., 1995a; Kwan et al., 1995a; Zhu et al., 1995; reviewed by Schwarz et al., 1996; Remold-O'Donnell et al., 1997; Zhu et al., 1997). However, in spite of the knowledge of WASP structure and mutations, the overall mechanism by which lack of WASP expression leads to the complex WAS phenotype remains unknown.

Studies of blood cells from human WAS patients have provided insights into potential functions of the WAS protein. T cell lines derived from WAS patients have defective responses to antigen receptor–induced stimulation in vitro. However, both calcium mobilization upon antigen receptor–induced stimulation and proliferative responses to compounds such as phorbol esters (phorbol myristate acetate or PMA) and calcium ionophores (ionomycin), which bypass receptor-proximal signaling events, appear to be normal in these cells (Molina et al., 1993). Together, these results suggested a role for WASP in T cell signal transduction downstream from the T cell receptor (TCR) but upstream from PMA and ionomycin targets (e.g., protein kinase C and Ca^{2+} mobilization, respectively). Additional studies showed that T lymphocytes from WAS patients contained reduced numbers and size of surface microvilli, consistent with a role for WASP in regulation of the actin cytoskeleton (Kenney et al., 1986; Molina et al., 1992). A direct connection between the aberrant TCR signaling and altered cytoskeleton in T cell lines from WAS patients came from the finding that such cells formed abnormal shapes upon antigen receptor–induced activation and failed to polymerize actin (Gallego et al., 1997). On the other hand, the role of WASP in B cell receptor signaling has not been clearly determined, with one report suggesting normal and another suggesting abnormal B cell receptor signaling in WAS-deficient human B cells (Simon et al., 1992; Henriquez et al., 1994).

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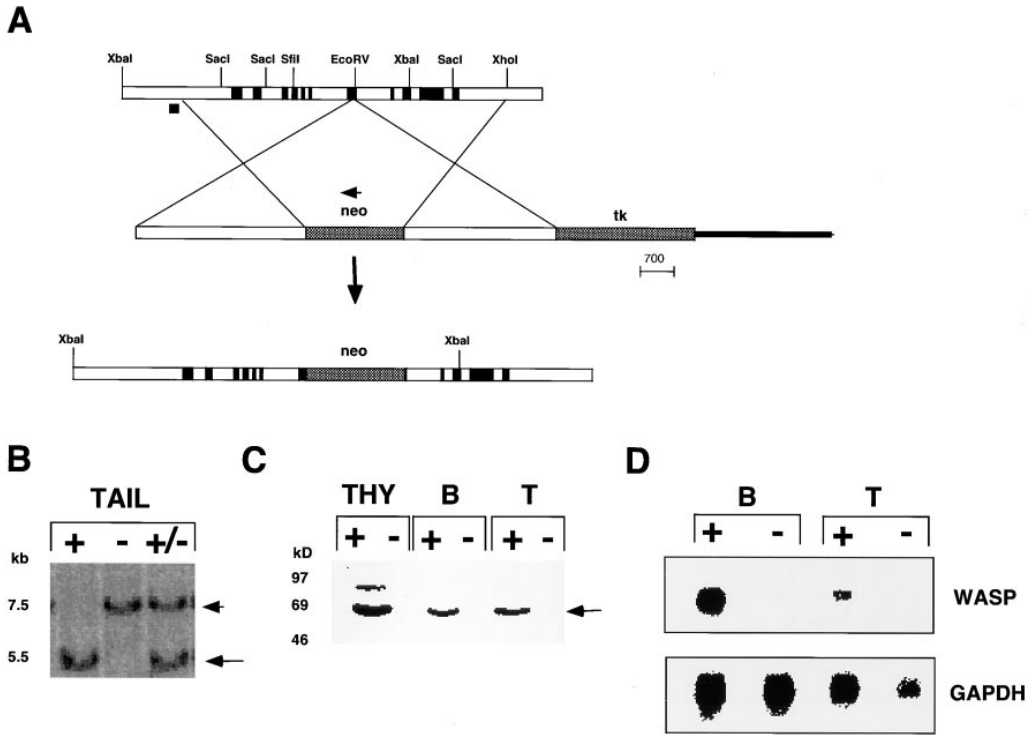

Figure 1. Targeted Disruption of the *WASP* Gene by Homologous Recombination

(A) Targeting strategy. Endogenous *WASP* locus (upper bar), targeting construct (middle bar), and targeted *WASP* locus (lower bar) are displayed. The first 11 (of 12) coding exons are shown by small black rectangles, with selected restriction sites denoted above bars. The neomycin-resistance (*neo*) and thymidine kinase (*tk*) genes used for positive and negative selection, respectively, are indicated. Probe used for screening ES transfectants is indicated with small filled rectangle under upper bar. Scale bar = 700 bp.

(B) Southern blot analysis of tail DNA isolated from male hemizygous $WASP^{+/}$ (+), $WASP^{-/}$ (−) and female heterozygous $WASP^{+/-}$ (+/−) mice. Using probe noted in targeting schematic, upon XbaI digestion a *neo* gene insertion by homologous recombination into the *WASP* locus results in the shift of a single 5.5 kb band (arrow) to a 7.5 kb band (arrowhead).

(C) Western blot analysis of protein extracts of thymocytes (THY), B cells (B), and T cells (T) from wild-type (+) and $WASP^{-/}/RAG2^{-/-}$ chimeric (−) mice. WASP runs at approximately 65 kDa (arrow) and is absent in lysates from lymphoid tissues isolated from $WASP^{-/}/RAG2^{-/-}$ chimeric mice. Fresh extracts from lymphoid tissues were fractionated by 8% SDS-PAGE. Equal protein loading on immunoblots was confirmed by Ponceau S staining. The immunoblot was stained using an immunoaffinity-purified rabbit anti-WASP antisera raised against a peptide from the C terminus of WASP. Identical results were obtained with an antibody raised against the N terminus (data not shown; Kolluri et al., 1996).

(D) Northern Blot analysis of RNA isolated from B and T cells from wild-type (+) and $WASP^{-/}$ (−) mice probed with a *WASP* cDNA (upper panel) or *GAPDH* control cDNA (lower panel) fragment. The murine *WASP* cDNA probe contained exons 1–6.

A significant clue to potential WASP functions came from the finding that WASP interacts in vivo, via the GBD domain, with CDC42 (Aspenstrom et al., 1996; Kolluri et al., 1996; Symons et al., 1996). CDC42 and other Rho family GTPases (Rac and Rho) are known to regulate the cytoskeleton (reviewed by Hall, 1998). In this context, CDC42 can directly induce the formation of filopodia and can indirectly induce the formation of lamellipodia and actin stress fibers through activation of Rac and Rho (Nobes and Hall, 1995). When overexpressed, WASP induces actin clustering and can prevent such CDC42-mediated cytoskeletal changes (Symons et al., 1996). In this regard, recent studies also have implicated a mammalian WASP homolog (N-WASP) and a yeast WASP-like protein (Bee1) in regulating the actin cytoskeleton (Miki et al., 1996; Li, 1997; Miki et al., 1998). In addition, several other molecules have been identified, including WIP (WASP-interacting protein) and the coiled-coil PSTPIP, that can both interact with WASP and regulate the actin cytoskeleton (Ramesh et al., 1997; Wu et al., 1998). WASP family proteins may, therefore, play a role in coordinating responses to external stimuli and initiating cytoskeletal changes by inducing actin reorganization directly or by acting as an adapter or scaffold for other signaling and structural molecules.

A clearer understanding of the functions of WASP will require elucidation of the signaling and cytoskeletal events that rely on its function. To gain further insight into the function of WASP, we have disrupted the *WASP* gene in murine embryonic stem cells, introduced this mutation into the murine germline, and then examined the effects of the mutation in WASP-deficient mice.

Results

Generation of Mice

The *WASP* gene is X-linked and, therefore, is present in only a single copy in the male TC-1 ES cell line (referred to as $WASP^{+/}$). To disrupt the murine *WASP* gene, we employed gene-targeted mutational techniques to insert a neomycin-resistance gene (*neo*) into exon 7 of *WASP* in the reverse transcriptional orientation in TC-1

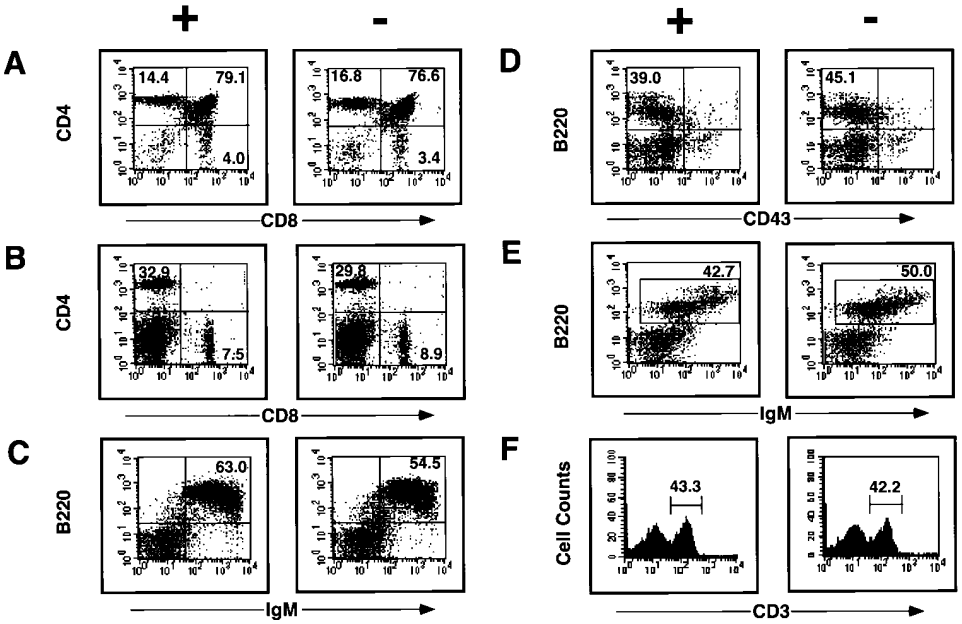

Figure 2. Normal Lymphoid Development in Wild-Type and *WASP*$^{-/}$ Mice

(A–F) FACS analyses of lymphocytes from wild-type (+) and *WASP*$^{-/}$ (−) mice. Two-color flow cytometric analyses was performed on thymocytes (A), spleen (B–C), and bone marrow (D–E). Percentages of representative lymphoid populations are noted. The histogram (F) demonstrates the surface CD3ε expression on splenic T lymphocytes. The results shown are representative of at least three male mice of each type analyzed at the age of 6–8 weeks.

cells (Figure 1A). This exon encodes a portion of the GTPase-binding domain (Aspenstrom et al., 1996; Kolluri et al., 1996; Symons et al., 1996). Three independent *WASP*-targeted (*WASP*$^{-/}$) ES cell clones were used to generate chimeric mice, and the offspring were bred to generate germline *WASP*$^{-/}$ male mice (Figure 1B). These *WASP*$^{-/}$ male mice were viable, of similar weight to their wild-type littermates, and were fertile. Preliminary analyses of WASP-deficient female mice (*WASP*$^{-/-}$) showed no significant differences with the *WASP*$^{-/}$ males, indicating that there are no genes on the Y chromosome that significantly alter this phenotype. Therefore, although most of our presented analyses represent studies of *WASP*$^{-/}$ male mice and lymphocytes, we will simply refer to all as WASP-deficient mice and lymphocytes. Likewise, we used the RAG2-deficient blastocyst complementation method (Chen et al., 1993) to generate *WASP*$^{-/}$ lymphocytes from the *WASP*$^{-/}$ ES cells; analyses of these lymphocytes in several studies (e.g., development, proliferation, and capping) revealed no differences from *WASP*$^{-/}$ lymphocytes from the germline mutant mice, indicating that observed defects are likely intrinsic to the lymphocytes.

Lymphocyte development occurred relatively normally in WASP-deficient mice (see below). Therefore, we were able to assess the effects of our targeted mutation on WASP expression by performing Western blotting analyses on protein extracts generated from lymphoid tissues of WASP-deficient and wild-type control mice. WASP was absent from extracts of thymus or from purified T and B cells from mutant animals, but it was readily detectable from those of wild-type animals (Figure 1C). Furthermore, WASP-hybridizing transcripts were not detectable when RNA from lymphoid tissues of WASP-deficient mice was assayed by Northern blotting techniques (Figure 1D). Therefore, we conclude that our targeted insertion into the *WASP* gene led to a complete loss of WASP RNA and protein expression.

Normal Lymphoid Development but Decreased Peripheral Blood Lymphocyte Numbers in WASP-Deficient Mice

T cell development in the thymus can be followed by the expression patterns of the CD4 and CD8 surface antigens. Immature double-negative thymocytes (CD4$^-$ CD8$^-$) give rise to double-positive thymocytes (CD4$^+$ CD8$^+$), which are the precursors of single-positive (SP) cells (either CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$), which can migrate to the peripheral organs such as the spleen and lymph nodes. B lineage cell development can be similarly followed by the presence of B220$^+$/CD43$^-$ and B220$^+$/IgM$^+$ pre-B cells in the bone marrow and B220$^+$/IgM$^+$ mature B cells in the periphery. Flow cytometric analyses of B and T lymphocytes in these various organs from 6- to 8-week-old WASP-deficient mice revealed the presence of normal percentages of the various thymic, T, and B lineage cell populations (Figures 2A–2E; data not shown). Furthermore, the numbers of cells in thymus, spleen, and lymph nodes (Table 1) were approximately normal in young mutant mice (Table 1). In addition, we observed relatively normal numbers of B1 cells in the peritoneum of WASP-deficient mice (data not shown). These findings indicate that WASP deficiency does not have a marked effect on overall lymphocyte development.

In contrast to the normal lymphocyte numbers in the

Table 1. Peripheral Blood Analysis and Overall Lymphoid Cellularity from Wild-Type (WT) and *WASP*$^{-/}$ Mice

	Peripheral Blood					Lymphoid Organs		
Mice	Platelets[a] (10^3)	Leukocytes[a] (10^3)	Lymphocytes[a] (10^3)	Neutrophils (10^3)	Hemoglobin (g/dl)	Thymus (10^6)	Spleen (10^6)	Lymph node (10^6)
WT	747 ± 65	5.9 ± 0.95	4.8 ± 0.90	0.62 ± 0.20	15.2 ± 0.36	78 ± 14	57 ± 18	17 ± 9.3
WASP$^{-/}$	527 ± 38	3.9 ± 0.81	2.6 ± 0.46	0.80 ± 0.20	14.9 ± 0.52	89 ± 29	41 ± 10	12 ± 3.1

[a] Statistically significant difference, $p < 0.005$ (Student t test).
Data presented are mean values ± 95% confidence interval (CI).

spleen and lymph nodes, the number of lymphocytes in the blood of WASP-deficient animals was significantly reduced compared with wild-type animals (Table 1). This lymphopenia was associated with slightly elevated numbers of neutrophils but normal numbers of red blood cells (Table 1). The decreases in B and T cell numbers in peripheral blood were comparable (data not shown); therefore, in the young WASP-deficient mice analyzed (less than 10 weeks old), there was no significant change in blood B to T cell ratios. We conclude that T and B cell development proceeds in an apparently normal fashion in WASP-deficient mice; however, for as yet unknown reasons, these animals do have reduced T and B cell numbers in their peripheral blood.

Reduced Platelet Numbers in WASP-Deficient Mice

WAS patients have platelets that are smaller and markedly decreased in number (Cooper et al., 1968; Ochs et al., 1980), often leading to frequent bleeding complications. Young WASP-deficient mice also had reduced numbers of platelets compared with wild-type mice (Table 1). However, in contrast to findings with WAS-deficient humans, these differences in platelet numbers, although significant, were modest and were not associated with reduced platelet size (data not shown) or clinical signs of bleeding.

Aberrant Antigen Receptor–Induced Proliferation in WASP-Deficient T Cells, but Not B Cells

To assess whether WASP deficiency results in T cell dysfunction, T cells from peripheral lymph nodes of mutant mice were tested for their ability to proliferate in response to anti-CD3ε-mediated antigen receptor stimulation. WASP-deficient T cells were markedly defective in their ability to proliferate following anti-CD3ε stimulation as compared to wild-type T lymphocytes (Figure 3A). This impairment was not due to decreased CD3ε surface expression, because the level of CD3ε expression on the surface of wild-type and mutant T cells was similar (Figure 2F). Proliferative responses to concanavalin A (Con A), a lectin that stimulates T lymphocytes by cross-linking surface receptors, were also diminished in WASP-deficient T cells compared to wild-type T cells. However, these latter differences were less pronounced when compared to those mediated via direct antigen receptor stimulation (Figure 3A). Notably, costimulation with anti-CD28 antibodies or exogenously added IL-2 markedly improved antigen receptor induced proliferative responses (Figure 3A); the latter finding is consistent with the observation that murine (data not shown) and human WAS T cells (Molina et al., 1993) are deficient in IL-2 secretion. The combination of PMA and ionomycin, which bypasses cell surface stimulation, led to normal levels of proliferation in mutant T lymphocytes (Figure 3A). Therefore, antigen receptor–induced proliferative responses are defective in WASP-deficient T cells, but CD28 costimulatory pathways and downstream signaling events remain relatively intact.

To study the role of WASP in antigen receptor–induced proliferative responses in B cells, we stimulated wild-type and WASP-deficient splenic B cells with anti-IgM antibodies. The antigen receptor–induced proliferative responses of wild-type and mutant B cells were indistinguishable (Figure 3B). Furthermore, stimulation by lipopolysaccharide or anti-CD40 also induced equal proliferative responses in wild-type and mutant B cells (Figure 3B). Therefore, in contrast to the result with T cells, these results indicate that WASP is not required for antigen receptor–induced proliferative responses in B cells.

Defective Antigen Receptor–Induced Capping in WASP-Deficient T Cells

To investigate potential roles of WASP in regulating the T cell cytoskeleton and to further explore potential mechanisms responsible for the defective antigen receptor–induced proliferation in T cells, we studied the ability of WASP-deficient T lymphocytes to form caps upon antigen receptor stimulation. The clustering of surface receptors upon receptor cross-linking ("capping"; Taylor et al., 1971) has been demonstrated to require actin polymerization (de Petris, 1974; Kammer et al., 1983). Wild-type and WASP-deficient T cells purified from lymph nodes were incubated in vitro in the presence or absence of stimulatory anti-CD3ε antibodies and then surface stained with anti-CD3ε antibodies following fixation. Antigen receptor capping was attenuated by more than 80% in WASP-deficient T cells (Figures 4C and 4D) as compared with wild-type T cells (Figures 4A and 4B). Similarly, to assay capping in B cells, we incubated splenic B cells from mutant and wild-type mice with anti-IgM antibodies. In contrast to T cells, antigen receptor–induced capping of WASP-deficient B cells (Figures 4G and 4H) was identical to that of wild-type B cells (Figures 4E and 4F). These results parallel the normal antigen receptor–induced B cell proliferation studies described above and suggest a direct relationship between defective proliferative responses and receptor capping in T cells.

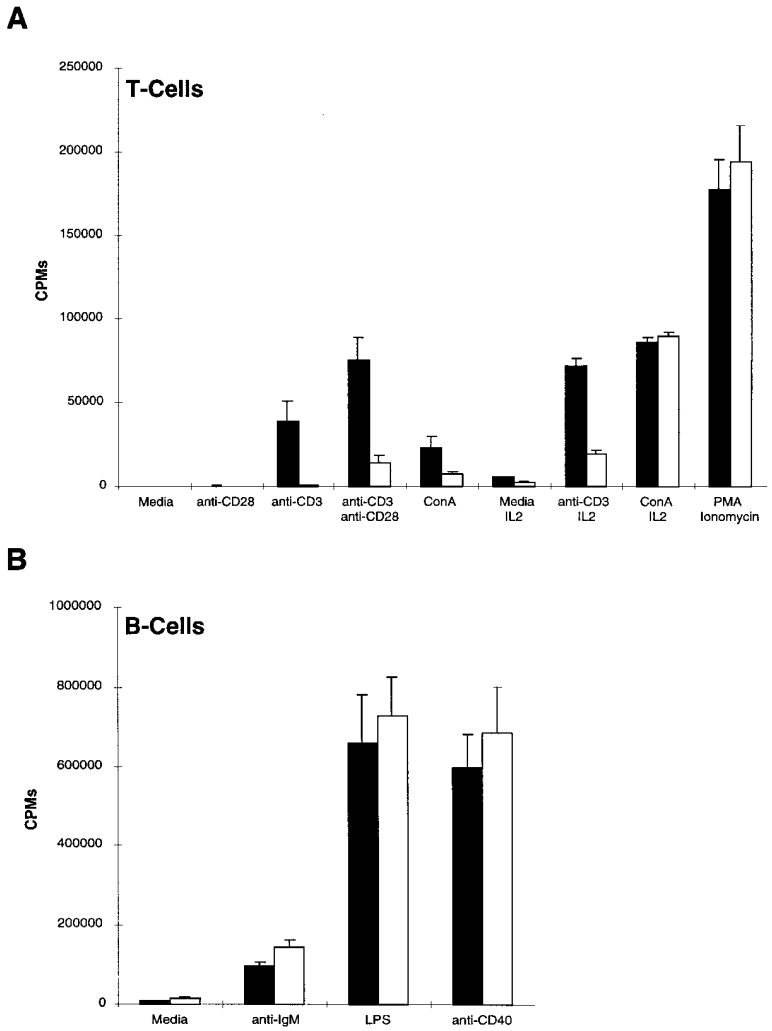

Figure 3. Defective Antigen Receptor–Induced Proliferation in WASP-Deficient T Cells, but Not B Cells

(A) T cell proliferation. Lymph node T cells from male *WASP*$^{-/}$ (empty bars) and age-matched wild-type (filled bars) mice were cultured in media alone or in the presence of stimulatory anti-CD3ϵ, anti-CD28, anti-CD3ϵ plus anti-CD28, or Con A. Each stimulation was performed in the absence or presence of exogenous IL-2 as indicated. Signaling pathways bypassing the surface were assessed by stimulation with phorbol myristate acetate (PMA) and ionomycin.

(B) B cell proliferation. Splenic B cells from male *WASP*$^{-/}$ (empty bars) and age-matched wild-type (filled bars) mice were cultured in media alone or in the presence of stimulatory anti-IgM (soluble), anti-CD40, or lipopolysaccharide (LPS). Each stimulation was performed in the presence of exogenous IL-4. In each experiment, cells were cultured in media for 48 hr, pulsed with 1 μCi [^{3}H]thymidine for an additional 16 hr, and then collected and scintillation counted. The data are displayed as raw cpm values. All assays were conducted in triplicate. Displayed are the results of one experiment. Each experiment was performed three times yielding similar results. (Raw cpm values for each of these three experiments are available in table form at http://www.immunity.com/supplemental/9/1/81).

WASP-Deficient Mice Are Capable of Mounting an Immune Response

Serum levels of immunoglobulin isotypes were determined by enzyme-linked immunosorbent assay (ELISA) in 6–8-week-old mutant mice and compared with wild-type littermates. WASP-deficient mice had normal levels of all Ig isotypes analyzed (Figure 5). To assess responsiveness of WASP-deficient mice to a T-dependent (TD) antigen, wild-type and mutant mice were immunized with TNP-Ova in incomplete Freund's adjuvant, and TNP-specific serum antibody titers were measured by ELISA. The immune responses to this protein antigen appeared relatively normal with respect to both total immunoglobulin (Figure 6A) and all tested serum Ig isotypes (data not shown). To investigate the response to T-independent type II (TI-II) carbohydrate antigens, wild-type and mutant mice were immunized with TNP-Dextran, and TNP-specific antibody titers were again determined by ELISA (Figure 6B). Both wild-type and WASP-deficient mice responded to this TI-II antigen. Therefore, serum Ig levels and antibody responses to a TD and a TI-II antigen in WASP-deficient mice appeared normal under these immunization conditions. These findings contrast with observations that humans with WAS have markedly decreased serum IgM and elevated IgA levels (Blaese et al., 1968; Cooper et al., 1968; Ochs et al., 1980) as well as poor antibody responses to carbohydrate and many protein antigens (Blaese et al., 1968; Cooper et al., 1968; Ochs et al., 1980).

Colitis in WASP-Deficient Mice

Unlike human WAS patients, none of the young WASP-deficient mice analyzed developed either hematopoeitic malignancies or eczema; although this will have to be further examined in older WASP-deficient mice. However, the majority of WASP-deficient mice developed chronic colitis by 4 months of age. The most severely involved colons were diffusely dilated with thickened walls and had marked mucosal thickening due to crypt hyperplasia and the presence of a mixed lymphocytic and neutrophilic infiltrate within the lamina propria (Figure 7). Crypt abscesses were present in colons of animals with severe colitis (Figure 7B). In contrast to wild-type mice, large numbers of CD4$^+$ T cells were scattered throughout the lamina propria in affected mutant mice

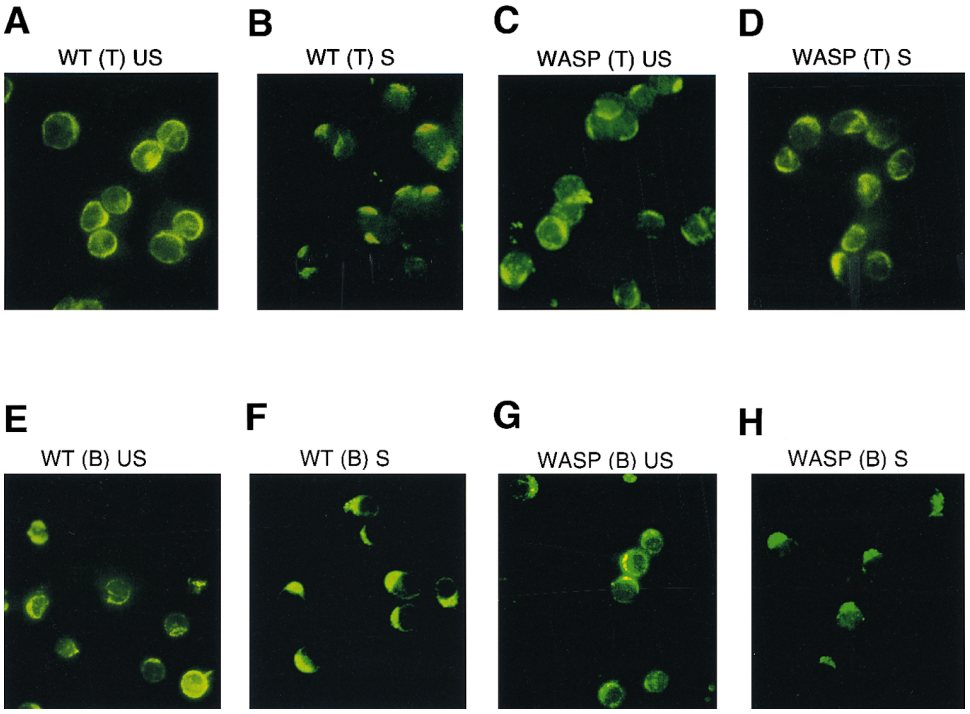

Figure 4. Aberrant Capping in *WASP*$^{-/}$ T, but not B, Lymphocytes

(A–D) T cell capping. Wild-type (A and B) and *WASP*$^{-/}$ (C and D) T cells were stimulated in the absence (A and C) or presence (B and D) of anti-CD3ε antibodies followed by immunostaining with FITC-conjugated anti-CD3ε antibodies. Caps were readily apparent in most wild-type cells stimulated through the antigen receptor. Significantly fewer caps were seen in similarly treated *WASP*$^{-/}$ T cells (<20% of wild-type) (D). (E–H) B cell capping. Wild-type (E and F) and *WASP*$^{-/}$ (G and H) B cells were stimulated in the absence (E and G) or presence (F and H) of anti-IgM antibodies followed by immunostaining with FITC-conjugated anti-IgM antibodies. Equivalent rates of antigen receptor–induced capping were seen in wild-type (F) and *WASP*$^{-/}$ (H) B cells.

(compare Figures 7C and 7D). There was a similar increase in CD8^{+} T cells in affected mice, but no increase in B220^{+} B cells (data not shown).

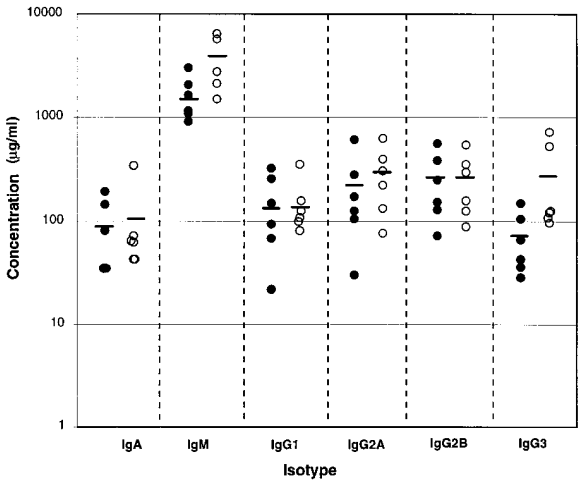

Figure 5. Normal Serum Immunoglobulin Levels in Wild-Type and *WASP*$^{-/}$ Mice

Serum immunoglobulin levels of specific isotypes were determined by ELISA in 6–8-week-old wild-type (filled circles) and WASP-deficient mice (open circles, six mice in each group).

Discussion

Comparison of the WASP-Deficient Phenotype in Mouse and Humans

In humans, the typical features of WAS are severe immunodeficiency (manifested as recurrent infections) plus eczema and decreased platelet size and numbers (thrombocytopenia). These features are usually associated with decreased numbers of blood lymphocytes (lymphopenia), lymphoreticular malignancies, defective T cell function, and poor immune responses to most protein antigens and all carbohydrate antigens (Remold-O'Donnell et al., 1996). Young WASP-deficient mice share many common features with WASP-deficient humans. In particular, they show mild thrombocytopenia and lymphopenia as well as defective T cell activation. However, none of the WASP-deficient mice analyzed have developed eczema or hematopoeitic malignancies. Likewise, their antibody responses to a TD antigen and a TI-II antigen appeared relatively normal. However, as the WASP-deficient animals have been maintained in a specific pathogen-free environment, it is not possible to fully assess their immunocompetence at this time; immunizations with other antigens or variations in the immunization protocol may show defects. In addition, it is possible that other missing aspects of the human phenotype might appear in older mice.

A striking difference between the human and murine

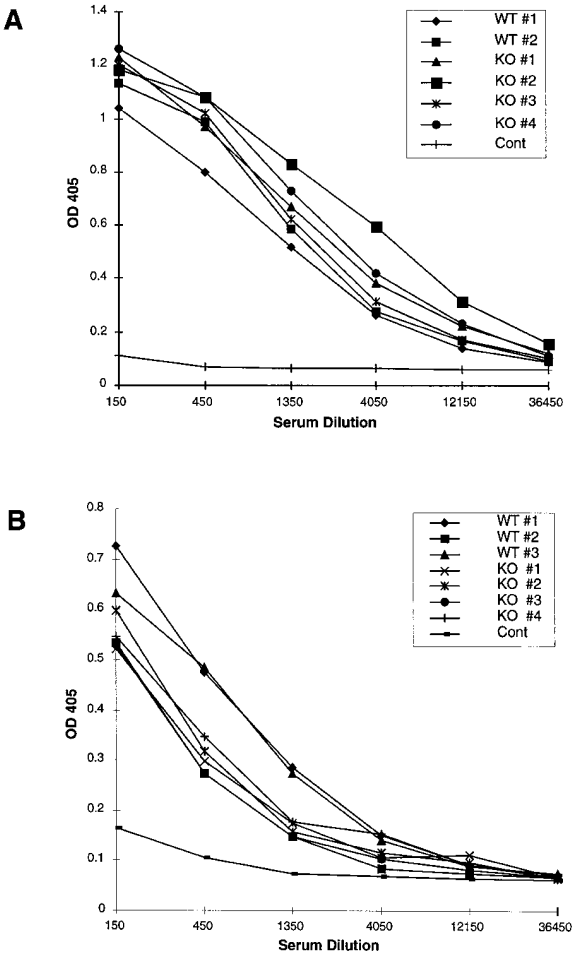

Figure 6. Immune Responses to TD and TI-II Antigens Are Similar between *WASP*$^{-/}$ and Wild-Type Mice

(A) Secondary antibody responses to TD antigen TNP-Ova. Secondary immune responses of wild-type and WASP-deficient mice were measured at 21 days, after primary footpad (day 1) and secondary subcutaneous (day 14) immunizations with 100 μg of TNP-Ova in incomplete Freund's adjuvant. A control wild-type littermate was similarly immunized with TNP-Ova in PBS. As above, total TNP-Ig was determined using serial dilutions of serum by ELISA with TNP-BSA as capture reagent.

(B) Antibody responses to TI-II antigen TNP-Dextran. Similar antibody responses were detected in wild-type (WT) and WASP-deficient (*WASP*$^{-/}$) mice 10 days following 50 μg intraperitoneal TNP-Dextran immunization. A sham-immunized wild-type littermate was used as control (CONT). Serum dilutions of serum were analyzed for TNP-specific total immunoglobulin by ELISA. Results are expressed as OD405 of anti-κ-specific ELISA using TNP-BSA as capture reagent.

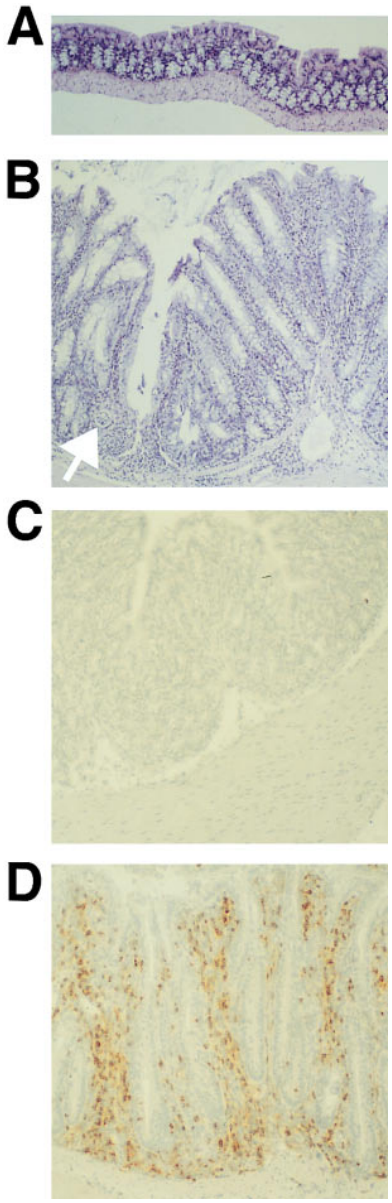

Figure 7. *WASP*$^{-/}$ Mice Develop Chronic Colitis

(A and B) Hematoxylin and eosin staining of colonic tissue from a wild-type (A) and *WASP*$^{-/}$ (B) mouse. The mucosa from affected WASP-deficient colons (B) was thickened with crypt hyperplasia and a lymphocytic and neutrophilic infiltrate in the lamina propria. Crypt abscesses were also present (arrow).

(C and D) Immunohistochemical analysis of colonic tissue from a wild-type (C) and *WASP*$^{-/}$ (D) mouse. CD4^{+} T cells were observed in the lymphocytic infiltrate in *WASP*$^{-/}$ mice (D). These cells are largely absent from wild-type colons (C). Magnification = 10×.

WASP-deficient phenotype is the consistent development of chronic colitis in WASP-deficient mice. Although bloody diarrhea is a feature of WAS in humans, inflammatory bowel disease has been reported only once in a WAS patient (Hsieh et al., 1988). Chronic colitis commonly develops in mice with mutations or other manipulations that alter T cell function or the ratio of T cell subsets (Kuhn et al., 1993; Mombaerts et al., 1993; Sadd-lack et al., 1993; Willerford et al., 1995; reviewed by Morales et al., 1996). Therefore, it is likely that the colitis observed in the WASP-deficient mice may be associated with their altered T cell function, although further analyses will be necessary to resolve this issue.

WASP Deficiency Affects Antigen Receptor–Induced Responses in T Cells, but Not B Cells

T cells from human WAS patients have diminished responses to antigen receptor stimulation and defects in

their actin cytoskeleton (Molina et al., 1993). However, the results with human WASP-deficient B cells have not been so clear, with some reports suggesting normal and others abnormal antigen receptor signaling (Simon et al., 1992; Henriquez et al., 1994). Our studies clearly demonstrate that, as in humans, T lymphocytes from WASP-deficient mice have major defects in antigen receptor–induced proliferation; our preliminary studies suggest that this is, at least in part, due to reduced progression through the cell cycle (S. B. S., unpublished data). In parallel, WASP-deficient T cells are markedly defective in their ability to form antigen receptor caps following anti-CD3ϵ stimulation, a process known to require actin polymerization (de Petris, 1974; Kammer et al., 1983). In contrast, WASP-deficient B cells proliferated normally in response to antigen receptor–induced stimulation, indicating that at least in mice, WASP is not required for antigen receptor–induced proliferation in B cells. Likewise, B cell capping was normal in WASP-deficient mice. Together, our studies suggest that there may be different requirements for WASP in antigen receptor signaling in B and T cells. In this context, one possibility is that the requirement(s) for cytoskeletal reorganization in antigen receptor signaling in T and B cells may be different. Alternatively, there may be functional redundancy in WASP-deficient B cells, perhaps mediated by a WASP-like protein such as N-WASP.

Potential Functions for WASP in Mediating TCR Responses

Potential insight into WASP-function may come from studies of Vav, a hematopoietic cell-specific Rho family GDP-GTP exchange factor that like WASP has also been shown to influence CDC42-mediated effects on the actin cytoskeleton in vivo (Olson et al., 1996). Targeted inactivation of Vav in lymphocytes results in a number of phenotypic similarities to WASP deficiency, including defective T cell proliferative responses and defective TCR capping following TCR/CD3 stimulation (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995; Fischer et al., 1998; Holsinger et al., 1998). To date, capping studies of Vav-deficient B cells have not been reported. Also, as in WASP deficiency, B cell development, peripheral B cell numbers, and Ig isotypes are normal in the context of Vav deficiency (Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995). However, there are significant differences in the two phenotypes, including a quantitative impairment in T cell development, lack of B1 cells, and impaired B cell receptor–induced proliferative responses of B cells in the context of Vav deficiency. Overall, the shared aspects of the Vav-deficient and WASP-deficient phenotypes are consistent with the possibility that Vav and WASP may be common members of a signal transduction pathway that regulates proliferation and capping upon antigen receptor activation. If so, differences in the two phenotypes might be attributed to redundant factors for WASP but not Vav in B cells and early T cells.

TCR activation has been directly linked to actin polymerization and cytoskeletal changes (Melamed et al., 1991; Pardi et al., 1992; Parsey and Lewis, 1993; Phatak and Packman, 1994; Selliah et al., 1995; Lowin-Kropf et al., 1998). Moreover, recent studies indicated that assembly of the actin cytoskeleton and capping may have a direct role in mediating signals from the TCR and further suggested that the proliferation defects in Vav-deficient T cells may result from impairment of these processes (Fischer et al., 1998; Holsinger et al., 1998). The close correlation between defects in proliferation and capping in WASP-deficient T versus B cells also suggests that these two processes may be directly related. Therefore, by analogy to the model for defects in Vav-deficient T cells (Fischer et al., 1998; Holsinger et al., 1998), the proliferation defects of WASP-deficient T cells may result from a defect in activation of a downstream signaling pathway dependent on the capping process. On the other hand, the proliferation defect may reflect an indirect role for WASP in T cell signaling. For example, altered cell shapes may prevent efficient contact in vitro between WASP-deficient T cells and immobilized anti-CD3ϵ antibodies.

Our results clearly demonstrate that WASP is critical for the readout of TCR-mediated signals that lead to proliferative responses. In this regard, the ability of anti-CD28 costimulation of WASP-deficient T cells to improve TCR-mediated proliferative responses indicates that such costimulatory signals somehow circumvent this block in TCR-mediated signaling. In addition, the apparently normal in vivo responses of WASP-deficient mice to a protein antigen may reflect the fact that in vivo T-dependent immune responses occur in the context of costimulatory pathways. Finally, because T cell development in the thymus also requires signaling through the CD3 complex and appears normal in WASP-deficient mice, signaling events in these earlier stage cells may occur despite WASP deficiency—for example, because of lower thresholds for activation or because of the presence of redundant factors.

Experimental Procedures

Targeted Inactivation

We cloned the murine homolog of the human *WASP* gene from a thymus B6/CBA F1 cDNA phage library (Stratagene, La Jolla, CA) using a human WASP cDNA (p427) as probe (Kwan et al., 1995b). The largest clone contained a 2048 bp insert that encoded an ORF of 520 amino acids. Our sequence was virtually identical to that published except for two amino acid (compared with the published sequence, our sequence had a glycine replacing an alanine at position 73 and an alanine replacing a valine at position 249) (Derry et al., 1995b). In contrast to the published sequence, all of our clones had poly(A) tails. To isolate *WASP* genomic sequences, a murine 129Sv λ phage genomic library was screened with the mouse *WASP* cDNA as probe. Several genomic clones were isolated and mapped by standard techniques. Two phage clones (numbers 3 and 8) contained approximately 15 kb of genomic sequence encompassing the entire *WASP* coding sequence and included over 1.5 kb upstream of the translational start site. A 7.5 kb genomic fragment from phage clone 8 spanning exons 1–11 (Derry et al., 1995b) contained within a NotI–XhoI fragment was subcloned into pBluescript (Stratagene). A targeting construct was developed with this plasmid by inserting a neomycin-resistance (*neo*^{r}) gene into a unique restriction site within the coding sequence of exon 7 (EcoRV) and a thymidine kinase (*tk*) gene at the 3′ end of the genomic sequence (within an XhoI site).

TC-1 ES cells (Deng et al., 1996) were electroporated with the targeting construct (pSBS55) and transfectants isolated after positive and negative selection following standard methodology (Mansour et al., 1988). To identify ES clones that contained targeted

recombination events within the *WASP* gene, DNA from transfectants was screened by Southern blotting procedures with a probe lying just outside of the targeted region. Targeted ES cells were used to inject C57BL/6 blastocysts; highly chimeric male mice were bred to C57BL/6 or 129Sv female mice to generate F1 offspring. Female $WASP^{-/+}$ offspring were bred to 129 wild-type male mice; 50% of male F2 mice were $WASP^{-/}$. Mating of $WASP^{-/}$ males with $WASP^{+/-}$ females yielded $WASP^{-/-}$ female mice at the expected frequency. While most of our analyses were performed with $WASP^{-/}$ male mice, analyses of a limited number of $WASP^{-/-}$ female mice gave similar results. For some analyses (i.e., lymphocyte development, antigen receptor activation, and capping), we also employed $WASP^{-/}$ T or B cells derived from chimeric mice generated by injecting $WASP^{-/}$ ES cells into *RAG2*-deficient blastocysts (*RAG2*-deficient blastocyst complementation; Chen et al., 1993). We found no differences in the analyses of WASP lymphocytes derived from either chimeric or germline $WASP^{-/}$ mice.

Peripheral Blood and Lymphoid Tissue Analyses

Platelet and leukocyte counts and red blood cell content were determined from fresh blood samples of 14 wild-type and 19 $WASP^{-/}$ mice by measurement on a Coulter counter. Total lymphocyte and neutrophil counts were determined by multiplying the leukocyte counts of individual mice by the percentages of each cell type determined by counting individual Wright-stained smears. Thymus, spleen, and lymph node cell counts were determined from wild-type and $WASP^{-/}$ mice with a hemocytometer after collecting organs and making single-cell suspensions. Splenocytes were counted following red blood lysis. Each set of lymph nodes included axillary, femoral, and submandibular lymph nodes. In several mice, FACS analysis confirmed similar percentages of B and T cells in wild-type and mutant spleen and lymph node.

FACS Analyses

Single-cell suspensions of lymphoid cells were prepared and stained with antibodies following standard procedures. Antibodies used were either phycoerythrin (PE)-, fluorescein (FITC)-, or Cy-Chrome-conjugates from PharMingen (San Diego, CA) and are labeled on the axis and discussed in the text.

Mice and Health Status

Mice were housed in the animal facility at Children's Hospital under specific pathogen-free conditions. Wild-type and WASP-deficient mice were tested at Charles River Laboratories (Wilmington, MA) for serology, parasitology, and bacteriology as described previously (Mombaerts et al., 1993). No potential infectious pathogens known to cause colitis were found in our animal facility except for *Helicobacter* spp., which were found in both wild-type and mutant mice. Some *Helicobacter* species have been associated with colitis in immunodeficient mice (Shomer et al., 1997; Ward et al., 1997).

Immunizations

To measure TI-II immune responses, wild-type and $WASP^{-/}$ mice were immunized intraperitoneally with 50 μg of TNP-Dextran in PBS. Serum was analyzed at day 10 for TNP-specific total immunoglobulin by ELISA. To measure TD immune responses, wild-type and $WASP^{-/}$ mice were immunized in the footpad with 100 μg of TNP-Ova in incomplete Freund's adjuvant on day 1 and boosted subcutaneously with 100 μg of TNP-Ova on day 14. Specific secondary anti-TNP total immunoglobulin responses were determined from serum on day 21 by ELISA.

ELISA

Immunol 1 plates (Dynatech) were coated with isotype-specific antibody (5 μg/ml) or TNP-BSA (100 μg/ml) for quantitating serum isotypes or TNP-specific antibodies, respectively. Diluted serum samples were incubated in coated plates and revealed by alkaline phosphatase–labeled secondary antibodies. Isotype concentrations were determined by comparing serum dilutions to known quantities of specific isotypes on a standard curve.

Histological Examination

Specimens were fixed in 10% buffered formalin and stained with hematoxylin and eosin. The severity of colitis (grade, 0–3) was determined according to the diagnostic criteria previously described (Mombaerts et al., 1993; Mizoguchi et al., 1997). Of mice greater than four months, 0/8 wild-type mice (0%) had evidence of disease, whereas 6/8 WASP-deficient mice (75%) had colitis.

Immunohistochemical Analysis

Specimens were embedded in OCT (optimal cutting temperature) compound (Miles Inc., Elkhart, IN) on dry ice. Sections (4 μm) of specimens were stained by an avidin–biotin complex method as described previously (Mombaerts et al., 1993).

Proliferation Analyses

T cells were purified from lymph nodes of WASP-deficient male mice (either $WASP^{-/}$ germline mice or $WASP^{-/}/RAG2^{-/-}$ lymphoid chimeras) or age-matched wild-type control mice by magnetic sorting and removal of B cells with anti-Ig-coated Dynabeads (Dynal) using standard procedures. The purity of the resulting population exceeded 95% as confirmed by FACS analysis. Stimulating antibodies (PharMingen) were allowed to bind to 96-well tissue culture plates at 37°C for 60 min (anti-CD3ε, 12 μg/ml; anti-CD28, 10 μg/ml). Cells (5×10^4) were added to each well and cultured at 37°C in RPMI 1640 medium supplemented with 15% fetal calf serum, 100 U ml^{-1} penicillin/streptomycin, and 50 mM 2-mercaptoethanol. Con A (Sigma) and IL-2 (PharMingen) were added to indicated cultures at concentrations of 5 μg/ml and 5 ng/ml, respectively. Signaling pathways bypassing the surface were assessed by stimulation with PMA (10 ng/ml) and ionomycin (0.5 mM). Equivalent results were obtained with mutant lymphocytes obtained from either germline $WASP^{-/}$ mice or $WASP^{-/}/RAG2^{-/-}$ lymphoid chimeras. B cell proliferations were performed in an identical fashion. Splenic B cells were purified by isolating $B220^+$ cells from WASP-deficient male mice (either $WASP^{-/}$ germline mice or $WASP^{-/}/RAG2^{-/-}$ lymphoid chimeras) or age-matched wild-type control mice using flow cytometry. The purity of the resulting population exceeded 95% as confirmed by FACS analysis. B cells were cultured in media alone or in the presence of stimulatory anti-IgM (soluble, 4 μg/ml; Southern Biotechnology), anti-CD40 (5 μg/ml; PharMingen), or lipopolysaccharide (10 μg/ml; Sigma). Each stimulation was performed in the presence of exogenous IL-4 (2 ng/ml, PharMingen). In each proliferation experiment, wild-type ($WASP^{+/}$) and $WASP^{-/}$ T or B cells were cultured for 48 hr, pulsed with 1 μCi [^{3}H]thymidine for an additional 16 hr, and then collected and scintillation counted.

Capping

Purified lymph node T cells were incubated at 37°C for 20 min in the absence or presence of both soluble (10 μg/ml) and plate-bound (10 μg/ml) anti-CD3ε antibodies (PharMingen), collected and cytospun onto poly-L-lysine coated glass slides, fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X, stained with FITC-conjugated anti-CD3ε (PharMingen), coverslips applied, and then immediately analyzed and photographed at 100× using a Zeiss fluorescent microscope. B cell capping experiments were performed with splenic B cells analogous to T cell capping experiments, except stimulating antibodies were anti-IgM (Southern Biotechnology) and staining antibodies were FITC-conjugated anti-IgM (PharMingen). The rate of capping of unstimulated and stimulated wild-type and $WASP^{-/}$ B and T cells was determined by three separate investigators (blinded to antibody stimulation and to cell type) who counted the number of caps in approximately 200 cells per experiment.

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