

# Defective nuclear translocation of nuclear factor of activated T cells and extracellular signal-regulated kinase underlies deficient IL-2 gene expression in Wiskott-Aldrich syndrome

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**Background:** Proliferation and IL-2 production in response to T-cell receptor ligation are impaired in patients with Wiskott-Aldrich syndrome (WAS). The transcription factors nuclear factor- $\kappa$ B (NF- $\kappa$ B), nuclear factor of activated T cells (NF-AT), and activating protein-1 (AP-1) play a critical role in IL-2 gene expression.

**Objective:** To investigate the mechanisms of impaired IL-2 production after T-cell receptor ligation in T cells deficient in WAS protein (WASP).

**Methods:** T cells from WASP<sup>-/-</sup> mice were stimulated with anti-CD3 and anti-CD28. Nuclear NF- $\kappa$ B, NF-AT, and AP-1 DNA-binding activity was examined by electrophoretic mobility assay. NF-ATp dephosphorylation and nuclear localization were examined by Western blot and indirect immunofluorescence. Phosphorylation of the mitogen-activated protein kinases Erk and Jnk, and of their nuclear substrates Elk-1 and c-Jun, was examined by Western blot. Expression of mRNA for IL-2 and the NF- $\kappa$ B-dependent gene A20 and of the AP-1 components c-fos and c-Jun was examined by quantitative RT-PCR.

**Results:** Nuclear translocation and activity of NF- $\kappa$ B were normal in T cells from WASP<sup>-/-</sup> mice. In contrast, NF-ATp dephosphorylation and nuclear localization, nuclear AP-1 binding activity, and expression of c-fos, but not c-Jun, were all impaired. Phosphorylation of Jnk, c-Jun, and Erk were normal. However, nuclear translocation of phosphorylated Erk and phosphorylation of its nuclear substrate Elk1, which activates the c-fos promoter, were impaired.

**Conclusion:** These results suggest that WASP is essential for NF-ATp activation, and for nuclear translocation of p-Erk, Elk1 phosphorylation, and c-fos gene expression in T cells. These defects underlie defective IL-2 expression and T-cell proliferation in WAS. (J Allergy Clin Immunol 2005;116:1364-71.)

**Key words:** WASP, IL-2 gene regulation, NF- $\kappa$ B, NF-AT, Erk, Jnk, c-fos, c-Jun

Wiskott-Aldrich syndrome protein (WASP) is the 502-amino acid product of the gene mutated in Wiskott-Aldrich syndrome (WAS), an X-linked immunodeficiency characterized by thrombocytopenia, moderate to severe bleeding disorder, eczema, and increased susceptibility to infections.<sup>1-3</sup> WASP is expressed only in hematopoietic cells and is the first identified member of an expanding family of proteins that includes N-WASP and Scar/WAVE (WASP-family verprolin homologous protein).<sup>4</sup> WASP is a multidomain protein that interacts with the WASP-interacting protein (WIP) via its N-terminal, with cell division cycle 42-guanine triphosphate (Cdc42-GTP) via its GTPase binding domain, with multiple src-homology domain 3 (SH3) domain-containing proteins via its proline-rich region, and with actin and the Arp2/3 complex via its verprolin homology/cofilin homology/acidic (VCA) domain. WASP exists in cells in a closed inactive conformation. Binding of Cdc42-GTP or of SH3 domain of proteins such as Nck, Grb2, and cortactin causes a conformational change in WASP, which allows the VCA domain to interact with and activate the Arp2/3 complex to regulate actin polymerization. In contrast, WIP prevents WASP activation.<sup>5,6</sup>

WASP plays a critical role in T-cell activation. T cells from patients with Wiskott-Aldrich syndrome and WASP-deficient mice fail to proliferate, secrete IL-2, and increase their F-actin content after ligation of the T-cell receptor (TCR).<sup>2,7,8</sup> The minimal IL-2 promoter region sufficient for IL-2 induction on TCR ligation is a stretch of DNA extending 300 bp upstream of the transcription start site. This region contains binding sites for nuclear factor- $\kappa$ B (NF- $\kappa$ B), nuclear factor of activated T cells (NF-AT), and activating protein-1 (AP-1) transcription factors. Loss of even 1 of these sites results in a dramatic decrease in overall promoter activity. Three of the 5 NF-AT elements in the IL-2 promoter are composite NF-AT/AP-1 binding sites. Cooperation between the 2 different transcription factors is essential for the stability and activity of the DNA binding complex.<sup>9</sup>

Of the 5 known NF-AT genes, NF-ATp (also designated as NF-AT1 or NF-ATc2) and NF-ATc (NF-AT2 or NF-ATc1) appear to play a particularly important role in IL-2 gene

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#### Abbreviations used

AP-1:	Activating protein-1
ATF:	Activating transcription factor
DAPI:	4'-6-Diamidino-2-phenylindole, dihydrochloride
EMSA:	Electrophoretic mobility shift assay
ERK:	Extracellular signal-regulated kinase
GOI:	Gene of interest
HK:	House keeping
NF-AT:	Nuclear factor of activated T cells
NF- $\kappa$ B:	Nuclear factor- $\kappa$ B
NK:	Natural killer
P/I:	Phorbol 12 myristate 13-acetate and ionomycin
TCR:	T-cell receptor
WASP:	Wiskott-Aldrich syndrome protein
WT:	Wild-type

induction during T-cell activation.<sup>10</sup> In resting cells, all NF-AT family members reside in the cytoplasm in their phosphorylated inactive form. On TCR ligation, there is a rise in intracellular free  $\text{Ca}^{++}$  with subsequent activation of the phosphatase calcineurin, which dephosphorylates NF-AT and causes its translocation to the nucleus.<sup>11</sup> *IL-2* expression is strongly inhibited by calcineurin inhibitors.<sup>12</sup> Furthermore, T cells lacking calcineurin or both NF-AT1 and NF-AT2 do not produce *IL-2* in response to TCR stimulation.<sup>13,14</sup> The AP-1 family of transcription factors consists of homodimers and heterodimers of Jun (c-Jun, JunB and JunD), fos (c-fos, FosB, Fra1 and Fra2), or activating transcription factor (ATF2, ATF3/liver regenerating factor [LRF1], B-ATF). c-fos and c-Jun are important for *IL-2* expression.<sup>15</sup> Mitogen-activated protein kinases such as extracellular signal-regulated kinase (Erk), Jnk, and p38 regulate AP-1 activation after TCR ligation.<sup>16</sup> TCR engagement also results in activation of the I- $\kappa$ B kinase complex, resulting in the phosphorylation of I- $\kappa$ B and its degradation. This releases NF- $\kappa$ B and allows it to translocate to the nucleus.<sup>17</sup> All 5 members of the NF- $\kappa$ B family—NF- $\kappa$ B1 (p50/p150), NF- $\kappa$ B2 (p52/p100), RelA (p65), c-Rel, and RelB—have been shown to play an important role in *IL-2* gene activation.<sup>17</sup>

Little is known about the mechanisms that underlie the failure of WASP-deficient T cells to produce *IL-2* after TCR ligation. We demonstrate that WASP is essential for NF-ATp dephosphorylation and nuclear localization, as well as for nuclear translocation of phosphorylated Erk and subsequent phosphorylation of its nuclear substrate Elk1, which upregulates expression of c-fos, an essential component of AP-1.

## METHODS

### Mice

The generation of WASP<sup>-/-</sup> mice has been described previously.<sup>8</sup> WASP<sup>-/-</sup> mice were backcrossed onto the 129Sv background for 5 generations. Mice were housed under pathogen-free conditions according to institutional regulations, and studies were performed in accordance with Children's Hospital policies and procedures.

## Antibodies

Phospho-Erk antibody is from Cell Signaling Technology (Beverly, Mass). Erk, phospho-Elk1, and lamin mAbs are from Santa Cruz Biotechnology (Santa Cruz, Calif). Elk1 antibody is from Abcam (Cambridge, Mass). Actin mAb is from Chemicon International (Temecula, Calif). The NF-ATp specific antibody T2B1 (raised against the C-terminal domain of NF-ATp) was donated by Dr A. Rao.<sup>18</sup> Phospho-Jnk antibody is from Biosource International (Camarillo, Calif). Jnk, phospho-c-Jun, and c-Jun antibodies are from Cell Signaling Technology.

## Cell preparation and stimulation

Splenocytes were suspended in RPMI-1640 medium supplemented with L-glutamine, penicillin/streptomycin, and 10% FBS. T cells were purified from spleens by using mouse T-cell enrichment columns (R&D Systems Inc, Minneapolis, Minn) and consisted of 90% to 95% CD3<sup>+</sup> cells. Cells were cultured at 2 to 5 × 10<sup>6</sup> cells/mL and stimulated with plate-bound anti-CD3 mAb (145-2C11; BD Bioscience, Pharmingen, San Jose, Calif) and anti-CD28 mAb (37.51; BD Bioscience, Pharmingen) at 5 μg/mL each. Alternatively, T cells were incubated in suspension with 5 μg/mL rat antimouse CD3 mAb (clone KT3; Serotec, Raleigh, NC) for 20 minutes on ice, followed by stimulation with 20 μg/mL goat antirat F(ab')<sub>2</sub> fragments (Jackson ImmunoResearch, West Grove, Pa) for the indicated times. As controls, T cells were stimulated with 20 ng/mL phorbol 12 myristate 13-acetate (Sigma Aldrich, St Louis, Mo) and 0.5 mmol/L ionomycin (Sigma Aldrich).

## RT-PCR analysis of *IL-2*, *A20*, *c-fos*, and *c-Jun* gene expression

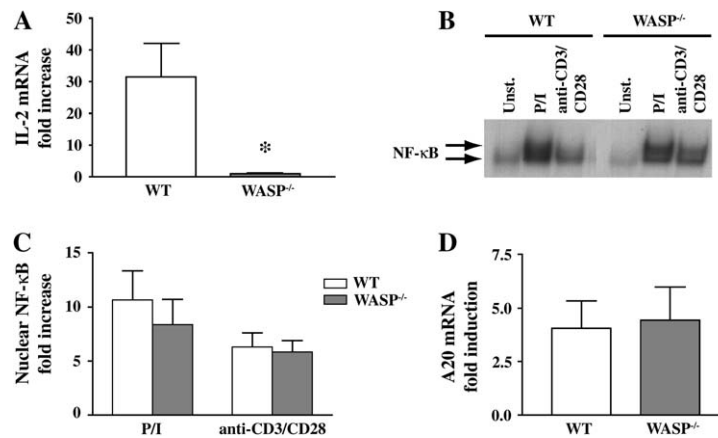
RNA was extracted from resting and stimulated T cells by using TRIzol (Invitrogen, Carlsbad, Calif). Total cDNA was prepared by using Superscript II RNA reverse transcriptase kit (Invitrogen), and *IL-2*, *A20*, *c-fos*, and *c-Jun* gene expression was analyzed by real-time PCR. Specific Taqman primers with 6-carboxyfluorescein (FAM)-labeled probes and 2x Taqman universal PCR master mix (Applied Biosystems, Foster City, Calif) were used as directed.<sup>19</sup> The relative expression ratio of the gene of interest (GOI) to the house keeping (HK) gene *β<sub>2</sub>-microglobulin* was calculated as previously described.<sup>20</sup> Fold induction was expressed as the GOI:HK ratio in stimulated cells divided by the GOI:HK ratio in unstimulated cells.

## Western blotting

One million cells were suspended in 30 μL cell lysis buffer that consisted of 50 mmol/L Tris pH 7.6, 150 mmol/L NaCl, 1% Nonidet P-40 (NP-40), 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L phenylarsine oxide, and protease inhibitor cocktail (P 8340; Sigma Aldrich) for 10 minutes on ice. The cells were then frozen in dry ice for 1 minute, vortexed at full speed for 10 seconds, then centrifuged 10 minutes at 14,000g and 4°C. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The indicated antibodies were diluted in TBS (10 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl) plus 5% milk and were used to probe the membranes. The blots were probed with antirabbit or antimouse antibodies linked to horseradish peroxidase (Bio-Rad, Hercules, Calif) and developed by using chemiluminescence (Amersham, Piscataway, NJ). To ensure equal loading, blots were stripped and then reprobed with the appropriate antibodies.

## Electrophoretic mobility shift assay

Cells were collected and nuclear extracts were prepared for electrophoretic mobility shift assay (EMSA) by using a Nuclear



**FIG 1.** *IL-2* mRNA expression, NF- $\kappa$ B translocation to the nucleus, and *A20* mRNA expression after T-cell stimulation with anti-CD3/CD28 antibodies. **A**, *IL-2* mRNA levels analyzed by real-time PCR in T cells stimulated for 6 hours. Results represent mean  $\pm$  SD fold increase of mRNA;  $n = 4$ ;  $*P = .03$ . **B**, EMSA analysis of NF- $\kappa$ B nuclear translocation in T cells stimulated 30 minutes as indicated. **C**, Quantitative analysis of EMSA results from 7 experiments. **D**, *A20* mRNA levels analyzed by real-time PCR in T cells stimulated for 30 minutes. Results represent mean  $\pm$  SD fold increase;  $n = 4$ . *Unst.*, Unstimulated.

Extract Kit from Active Motif (Carlsbad, Calif). The protein concentration was estimated by the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill). Single-stranded oligonucleotides were 5' end-labeled with [<sup>32</sup>P]-ATP by using T4-polynucleotide kinase, annealed, and purified on 12% PAGE in 1xTris borate EDTA buffer. For each reaction,  $1 \times 10^3$  cpm (0.1 ng) radiolabeled oligonucleotide probe was incubated with 5  $\mu$ g nuclear extracts in 20  $\mu$ L binding buffer (10 mmol/L Tris-HCl pH 7.5, 50 mmol/L NaCl, 5% glycerol, 50 ng/mL poly(dI-dC), 1 mmol/L EDTA, 0.1% NP-40, 1 mmol/L dithiothreitol, and the protease inhibitor mixture) for 30 minutes on ice or at room temperature. Samples were then run on 5% polyacrylamide gel in 1xTBE. The sequences of the oligonucleotides used were the following: NF- $\kappa$ B site of murine *IL-2* promoter, GAGGGATTTCACCTAAAT; consensus murine AP-1 DNA binding site, CGCTTGATGAGTCAGCCG; and murine *IL-4* P1 NF-AT site sequence, AATAAAATTTTCCAATGT. Radiographic signals were quantified by densitometry using NIH Image 1.62 (National Institute of Mental Health, Bethesda, Md). The signal found after stimulation was normalized to the unstimulated state.

### Immunofluorescence

T cells were spun onto poly-L-lysine-coated coverslips and fixed immediately in 3% paraformaldehyde. Cells were washed 3 times in wash buffer (1  $\times$  PBS, 0.5% NP-40, and 0.01% NaN<sub>3</sub>) and preincubated with wash buffer containing 10% FCS for 30 minutes. Cells were then incubated with rabbit anti-NF-ATp antibody for 45 minutes at room temperature followed by Cy3-conjugated sheep anti-rabbit IgG (Sigma Aldrich). The nuclei were stained with 1  $\mu$ g/mL 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Eugene, Ore). Cells were visualized with an Axiovert S200 epifluorescence microscope (Zeiss, Thornwood, NY) by using Openlab digital imaging software (Improvision, Lexington, Mass). Localization of NF-ATp was compared with that of DAPI nuclear staining in 150 to 300 cells for each sample.

### Statistical analysis

Statistical analysis was performed by using PRISM (GraphPad Software, San Diego, Calif) software. Wilcoxon test was used to compare the differences between groups. A *P* value less than .05 was considered statistically significant.

## RESULTS

### *IL-2* mRNA expression after TCR ligation is severely diminished in WASP<sup>-/-</sup> T cells

To determine whether the defect in *IL-2* synthesis was a result of impaired *IL-2* gene transcription, T cells from WASP<sup>-/-</sup> mice were stimulated with anti-CD3/CD28 mAbs for 6 hours, and *IL-2* mRNA levels were measured by real-time PCR. *IL-2* mRNA levels were significantly lower in T cells from WASP<sup>-/-</sup> mice compared with wild-type (WT) controls (Fig 1, A). This suggests that WASP is essential for *IL-2* gene expression after TCR ligation.

### NF- $\kappa$ B translocation and activation are normal in T cells from WASP<sup>-/-</sup> mice

Electrophoretic mobility shift assay was used to examine the presence of NF- $\kappa$ B in nuclear extracts from WASP<sup>-/-</sup> and WT T cells stimulated for 2 hours with anti-CD3/CD28 antibodies, or with phorbol 12 myristate 13-acetate and ionomycin (P/I). There was no significant difference in NF- $\kappa$ B nuclear binding activity between T cells from WASP<sup>-/-</sup> mice and WT controls (Fig 1, B). No difference was observed with shorter stimulations of 15 and 30 minutes (data not shown). Retarded bands were quantified by densitometry, and results were expressed as fold induction compared with unstimulated cells (Fig 1, C). Supershift assays revealed that the retarded bands in nuclear extracts of both WT and WASP<sup>-/-</sup> T cells contained the p50 and p65 subunits of NF- $\kappa$ B (data not shown).

T-cell receptor ligation induces NF- $\kappa$ B-dependent expression of the antiapoptotic gene *A20*.<sup>21</sup> There was no significant difference in the expression of *A20* mRNA levels between T cells from WASP<sup>-/-</sup> mice and WT controls stimulated 30 minutes with anti-CD3/CD28 antibodies, as assessed by real-time PCR (Fig 1, D).

Taken together, these results suggest that TCR ligation induces normal NF- $\kappa$ B nuclear translocation and activation in WASP<sup>-/-</sup> T cells.

### Impaired NF-AT activation in WASP<sup>-/-</sup> T cells

The DNA binding activity of NF-AT, assessed by EMSA on nuclear extracts of anti-CD3/CD28-stimulated T cells, was found to be significantly impaired in T cells from WASP<sup>-/-</sup> mice compared with WT controls (Fig 2). Supershift assays revealed that the retarded bands in nuclear extracts of WT T cells contained NF-ATp and NF-ATc (data not shown). Nuclear NF-AT binding activity was intact in WASP<sup>-/-</sup> T cells stimulated with P/I, agents that bypass TCR signaling (Fig 2).

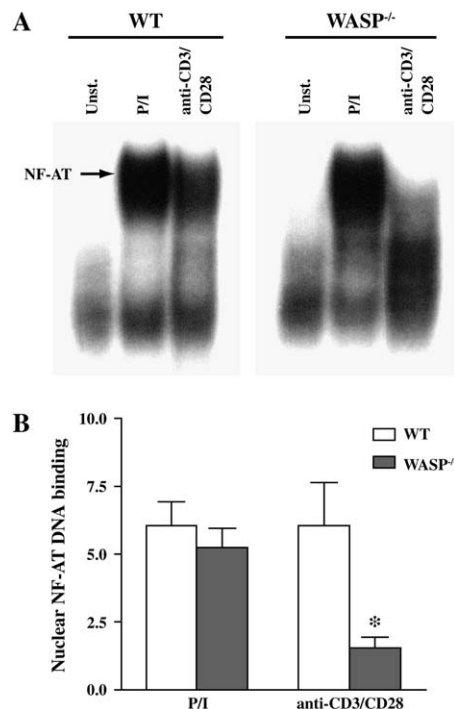
Dephosphorylation of the constitutively expressed NF-ATp after TCR ligation was examined by Western blot analysis of T cell lysates. Fig 3, A, reveals the appearance of a lower molecular weight band in WT T cells after stimulation with anti-CD3/CD28 mAbs, but not in WASP<sup>-/-</sup> T cells. NF-ATp was normally dephosphorylated in WASP<sup>-/-</sup> T cells after ionomycin stimulation. We also examined the dephosphorylation-dependent nuclear localization of NF-ATp by immunofluorescence. Fig 3, B and C, shows that NF-ATp is located in the nucleus of WT cells 30 minutes and 2 hours after stimulation with anti-CD3/CD28 mAbs (nuclear NF-ATp in 73% and 85% of the cells, respectively). In contrast, nuclear translocation of NF-ATp was impaired in WASP<sup>-/-</sup> T cells 30 minutes as well as 2 hours after stimulation with anti-CD3/CD28 mAbs (nuclear NF-ATp in 14% and 34% of the cells, respectively). However, nuclear translocation of NF-ATp was intact in WASP<sup>-/-</sup> T cells stimulated with ionomycin. These results show that WASP is essential for TCR-mediated induction of NF-ATp dephosphorylation and nuclear translocation.

### Impaired AP-1 DNA binding activity in WASP<sup>-/-</sup> T cells

Expression and activation of c-fos and c-Jun, 2 important components of the AP-1 complex,<sup>22</sup> are induced after TCR ligation.<sup>23</sup> The DNA binding activity of AP-1, assessed by EMSA on nuclear extracts of anti-CD3/CD28-stimulated T cells, was found to be significantly impaired in T cells from WASP<sup>-/-</sup> mice compared with WT controls (Fig 4). Supershift assays revealed that the retarded bands in nuclear extracts of WT T cells contained c-fos and c-Jun (data not shown). Nuclear AP-1 binding activity was intact in WASP<sup>-/-</sup> T cells stimulated with P/I. These results suggest that WASP is essential for TCR induction of AP-1 binding activity.

### Phosphorylation of Jnk and c-Jun, and c-Jun mRNA induction are not affected in WASP<sup>-/-</sup> T cells

T-cell receptor ligation causes phosphorylation and activation of Jnk, resulting in phosphorylation and activation of its nuclear substrate c-Jun.<sup>23</sup> Western blot analysis shows that TCR induction of Jnk phosphorylation is

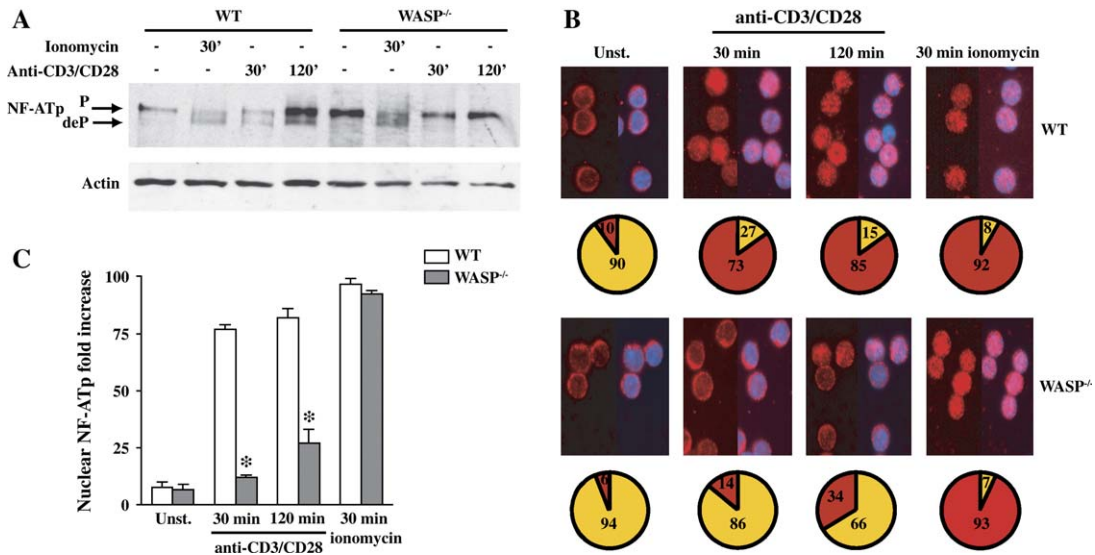


**FIG 2.** Impaired NF-AT DNA binding activity in WASP<sup>-/-</sup> T cells. **A,** Representative EMSA analysis of NF-AT DNA binding activity. T cells were stimulated 30 minutes as indicated. **B,** Quantitative analysis of EMSA. Results represent mean  $\pm$  SD from 7 experiments. \* $P = .03$ . *Unst.*, Unstimulated.

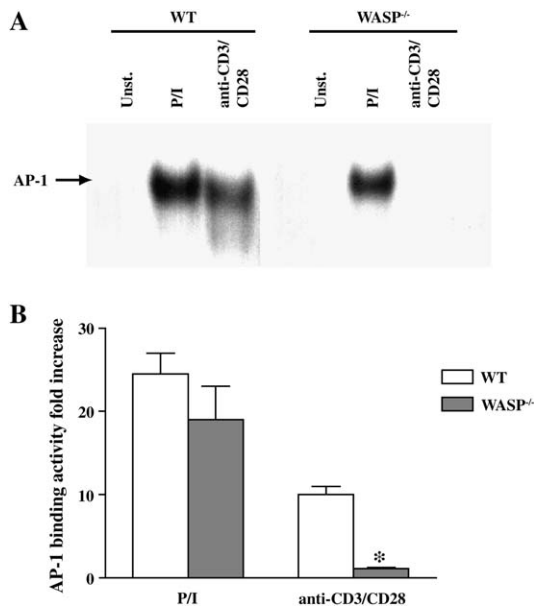
normal in WASP<sup>-/-</sup> T cells (Fig 5, A), as previously reported.<sup>7</sup> More importantly, phosphorylation of c-Jun, and upregulation of *c-Jun* mRNA expression after TCR ligation, were also normal in WASP<sup>-/-</sup> T cells (Fig 5, B and C). These results suggest that WASP is not important for Jnk phosphorylation and phosphorylation of its nuclear target c-Jun.

### Nuclear translocation of p-Erk, phosphorylation of Elk1, and induction of c-fos are defective in WASP<sup>-/-</sup> T cells

T-cell receptor ligation causes phosphorylation and activation of Erk and its translocation to the nucleus, where it phosphorylates and activates its nuclear substrate Elk1.<sup>24</sup> Activated Elk1 regulates the transcription of *c-fos*.<sup>16</sup> Western blot analysis showed that Erk1 and Erk2 phosphorylation was comparable in WT and WASP<sup>-/-</sup> T cells after TCR ligation, as previously described.<sup>7</sup> In contrast, Elk1 phosphorylation was diminished in WASP<sup>-/-</sup> T cells compared with WT controls (Fig 6, A). To investigate whether reduced Elk1 phosphorylation was a result of a defect in nuclear translocation of Erk, nuclear extracts were analyzed by Western blot using p-Erk specific antibodies. TCR stimulation resulted in the translocation of p-Erk1 and p-Erk2 into the nucleus of WT cells, but not of WASP<sup>-/-</sup> T cells (Fig 6, B). In addition, real-time PCR showed a significant decrease in *c-fos* mRNA induction in stimulated WASP<sup>-/-</sup> T cells



**FIG 3.** Defective NF-ATp dephosphorylation and translocation to the nucleus of WASP<sup>-/-</sup> T cells. **A**, T cells from WT and WASP<sup>-/-</sup> mice were stimulated and NF-ATp dephosphorylation determined by Western blot. Similar results were obtained in 3 experiments. **B**, T cells from WT and WASP<sup>-/-</sup> mice were stimulated, and NF-ATp was detected as described in Methods (red). Nuclei of the same cells were counterstained with DAPI (blue). Left panels show NF-ATp staining alone; right panels show merged images (NF-ATp and DAPI) for the same cells. Pie charts show percent of cells with cytoplasmic (yellow) and nuclear (red) NF-ATp staining. **C**, Pooled results of NF-ATp nuclear translocation from 3 experiments. Columns and bars represent means  $\pm$  SDs. \* $P = .03$ . Unst., Unstimulated; P, phosphorylated; Dep, dephosphorylated.



**FIG 4.** Impaired AP-1 DNA binding activity in WASP<sup>-/-</sup> T cells. **A**, Nuclear extracts from unstimulated WT and WASP<sup>-/-</sup> T cells, or cells stimulated 30 minutes, as indicated, were used in EMSA. **B**, Quantitative analysis of EMSA. Results represent means  $\pm$  SDs from 7 independent experiments. \* $P = .03$ . Unst., Unstimulated.

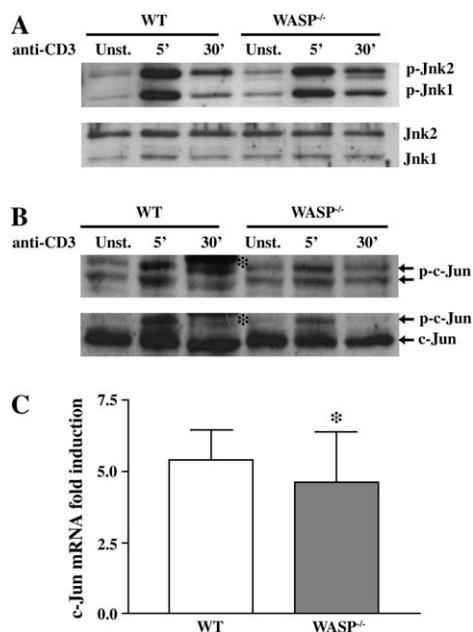
## DISCUSSION

The results of the current study show that WASP is essential for the proper activation of NF-AT and AP-1. We found that WASP is important for the dephosphorylation and nuclear translocation of NF-ATp, p-Erk nuclear translocation, Elk1 phosphorylation, and *c-fos* expression after TCR engagement. These abnormalities are likely to underlie the defective *IL-2* gene expression in WASP-deficient T cells after TCR ligation.

In agreement with a recent report,<sup>25</sup> we demonstrate that *IL-2* mRNA expression is virtually absent in T cells from WASP<sup>-/-</sup> mice (Fig 1, A). Translocation of NF- $\kappa$ B to the nucleus is normal in stimulated WASP<sup>-/-</sup> T cells (Fig 1, B and C). Furthermore, transcription of the NF- $\kappa$ B-dependent gene *A20*, which requires p50, p65, and c-Rel,<sup>21</sup> was normal in WASP<sup>-/-</sup> T cells (Fig 1, D), suggesting that the activities of all 3 NF- $\kappa$ B subunits are normal and do not depend on WASP for activation. Rel-A, another member of the NF- $\kappa$ B complex, was recently found to translocate to the nucleus of stimulated WASP<sup>-/-</sup> natural killer (NK) cells,<sup>26</sup> suggesting that the independence of NF- $\kappa$ B nuclear translocation from WASP is not restricted to a single cell type.

DNA binding activity of NF-AT was severely impaired in WASP<sup>-/-</sup> T cells (Fig 2). Immunofluorescence analysis showed that NF-ATp nuclear localization is defective in stimulated WASP<sup>-/-</sup> T cells (Fig 3). A defect in the nuclear translocation of NF-ATc, which is induced by activated NF-ATp, has been recently described in WASP<sup>-/-</sup> T cells.<sup>27</sup> However, the mechanism of this defective

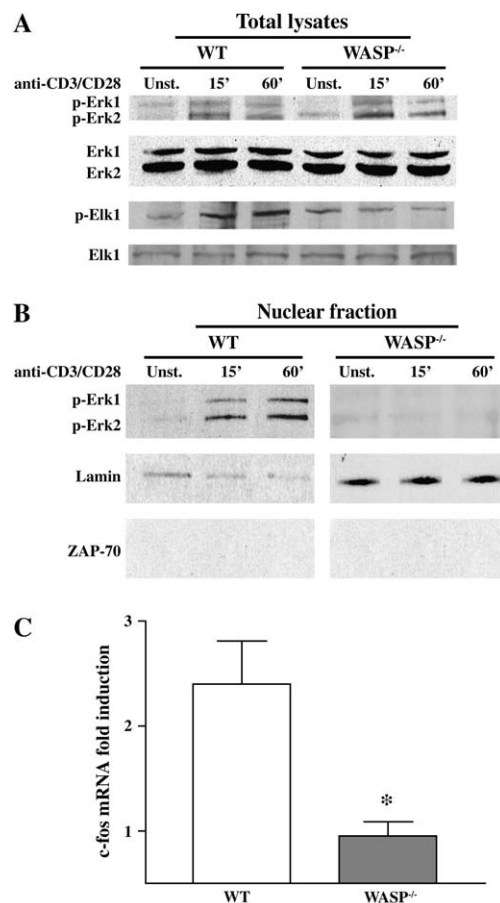
compared with stimulated WT T cells (Fig 6, C). These results show that WASP is essential for nuclear translocation of p-Erk, and consequently, for Elk1 phosphorylation and induction of *c-fos* expression.



**FIG 5.** Jnk and c-Jun phosphorylation and *c-Jun* mRNA induction are normal in WASP<sup>-/-</sup> T cells. **A and B,** Cell extracts from CD3-stimulated T cells were probed for p-Jnk and Jnk (**A**) as well as p-c-Jun and c-Jun (**B**). The results shown are representative of 3 different experiments. The p-c-Jun antibody detected 2 discrete bands, which may represent differentially phosphorylated forms of c-Jun. The upper band in the c-Jun blot (*lower panel*) that appears 5 minutes after stimulation, then fades at 30 minutes, is the phosphorylated c-Jun, and it corresponds to the upper band in the p-c-Jun blot (*upper panel*). The star (\*) indicates a nonspecific band. Results are representative of 2 experiments. **C,** *c-Jun* mRNA levels analyzed by real-time PCR in T cells stimulated with anti-CD3/CD28 mAbs for 30 minutes. n = 4. \*P = .03. *Unst.*, Unstimulated.

translocation has not been elucidated. Our results clearly demonstrate that dephosphorylation of NF-ATp, which is essential for its nuclear translocation,<sup>11</sup> is defective in WASP<sup>-/-</sup> T cells (Fig 3). The partial defect in calcium mobilization observed in WASP<sup>-/-</sup> T cells early (as long as 15 minutes) after TCR ligation<sup>7</sup> very likely results in reduced calcineurin activation and NF-ATp dephosphorylation. Defective nuclear localization of NF-ATp may be a result of defective nuclear entry or increased rate of exit from the nucleus. It is unlikely that enhanced exit from the nucleus would account for this defect, because 93% of WASP<sup>-/-</sup> T cells treated with ionomycin show nuclear localization of NF-ATp after 30-minute stimulation. WASP<sup>-/-</sup> NK cells show transient delay in NF-ATc translocation to the nucleus,<sup>26</sup> whereas the delay in the nuclear translocation of NF-ATp in WASP<sup>-/-</sup> T cells persisted as long as 2 hours. This difference may be explained by differences in the cell types and stimuli used (cellular targets for NK cells and anti-CD3/anti-CD28 in T cells).

Binding activity of AP-1 after CD3/CD28 ligation was severely impaired in WASP<sup>-/-</sup> T cells (Fig 4). After TCR ligation, phosphorylation of Jnk and of its nuclear target, *c-Jun*, as well as *c-Jun* mRNA upregulation, were all



**FIG 6.** Impaired nuclear translocation of p-Erk, phosphorylation of Elk1, and induction of *c-fos* gene expression in WASP<sup>-/-</sup> T cells. **A,** Lysates of T cells were probed with p-Erk, Erk, p-Elk1, and Elk1 antibodies. Results are representative of 2 experiments. **B,** p-Erk in the nuclear fraction of T cells. The blots were stripped and reprobed for ZAP-70 and lamin as markers for cytoplasmic and nuclear fractions. Results are representative of 2 experiments. **C,** Analysis of *c-fos* mRNA levels by real-time PCR in T cells stimulated with anti-CD3/CD28 mAbs for 30 minutes. n = 4. \*P = .03. *Unst.*, Unstimulated; ZAP-70, zeta chain (TCR) associated protein kinase 70.

normal in WASP<sup>-/-</sup> T cells (Fig 5). The normal upregulation of *c-Jun* mRNA expression in WASP<sup>-/-</sup> T cells (Fig 5, C), in spite of defective *c-fos* expression (Fig 6, C), may be explained by the fact that c-Jun homodimers and c-Jun/ATF2 heterodimers can activate the *c-Jun* promoter independently of *c-fos*.<sup>22,28</sup>

c-Fos is the limiting component of AP-1 in the induction of *IL-2* gene expression and is poorly expressed in resting T cells. Transcription of *c-fos* depends on the formation of a complex that contains serum response factor and a member of the Ets transcription factor family that includes Elk-1. This complex binds to the serum response element present in the *c-fos* promoter and activates transcription.<sup>16,28</sup> *c-Fos* expression after TCR ligation depends on the phosphorylation of Elk1 by the mitogen-activated protein kinase Erk, which translocates to the

nucleus after it phosphorylation,<sup>29</sup> We found that Erk was phosphorylated normally in WASP<sup>-/-</sup> T cells, but its nuclear translocation was defective. Furthermore, Elk1 phosphorylation and upregulation of *c-fos* mRNA expression were defective in WASP<sup>-/-</sup> T cells (Fig 6). These results suggest that WASP is important for nuclear translocation of activated p-Erk after TCR ligation. We cannot, however, rule out the possibility that the rate of exit of p-Erk from the nucleus is increased in WASP<sup>-/-</sup> T cells. T cells from mice with disrupted *Erk-1* gene exhibit poor proliferation to TCR ligation.<sup>30</sup> Defective nuclear translocation of p-Erk is likely to underlie the defects in Elk1 phosphorylation, *c-fos* upregulation, and AP-1 activation observed in WASP<sup>-/-</sup> T cells.

Although ionomycin activates NF-ATp normally in WASP<sup>-/-</sup> T cells, we found that addition of ionomycin to anti-CD3-stimulated T cells over a wide dose range (10-500 nmol/L) failed to reverse the defect in proliferation and IL-2 secretion of WASP<sup>-/-</sup> T cells (unpublished data, July 2005). This suggests that the NF-AT defect does not account by itself for the defective response of WASP<sup>-/-</sup> T cells to TCR ligation, and that the failure of p-Erk to translocate to the nucleus of WASP<sup>-/-</sup> T cells is likely to play an important role. This is supported by the observation that NF-AT binds to the *IL-2* promoter weakly and needs AP-1 for high-affinity binding and efficient transcriptional activation.<sup>9,31</sup> There is a similar dependence on both NF-AT and AP-1 for TNF- $\alpha$  production in mast cells after Fc $\epsilon$ RI ligation.<sup>32-34</sup> However, in addition to NF-AT and AP-1, TNF- $\alpha$  production by mast cells also requires a NF- $\kappa$ B-like nuclear binding complex.<sup>35</sup>

Disruption of actin polymerization with cytochalasin D does not affect NF- $\kappa$ B or NF-AT translocation to the nucleus.<sup>36,37</sup> In contrast, nuclear translocation of p-Erk is thought to require intact actin cytoskeleton because treatment with cytochalasin-D inhibits anchorage-dependent nuclear translocation of p-Erk and phosphorylation of its nuclear target Elk1 in serum-stimulated fibroblasts.<sup>38</sup> There are conflicting data about whether the actin polymerization activity of WASP is important for the NF-AT/AP-1-mediated *IL-2* gene transcription.<sup>27,39</sup> However, even if actin polymerization is not involved in NF-AT/AP-1 activation, an intact cytoskeleton may be essential.<sup>40</sup> It is possible that disruption of the actin dynamics caused by the absence of WASP may contribute to the defect in the nuclear translocation of p-Erk, leading to defective activation of AP-1.

It has been shown that N-WASP possesses nuclear localization and export signals and that it functions in the nucleus by modulating the expression of heat shock protein 90.<sup>41</sup> Because of the sequence similarity between N-WASP and WASP, this raises the question whether WASP translocates in and out of the nucleus and whether it acts as a chaperone for factors such as NF-AT and p-Erk.

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