Wiskott-Aldrich syndrome (WAS) is a primary immunodeficiency associated with an increased susceptibility to herpesvirus infection and hematologic malignancy as well as a deficiency of NK cell function. It is caused by defective WAS protein (WASp). WASp facilitates filamentous actin (F-actin) branching and is required for F-actin accumulation at the NK cell immunological synapse and NK cell cytotoxicity ex vivo. Importantly, the function of WASp-deficient NK cells can be restored in vitro after exposure to IL-2, but the mechanisms underlying this remain unknown. Using a WASp inhibitor as well as cells from patients with WAS, we have defined a direct effect of IL-2 signaling upon F-actin that is independent of WASp function. We found that IL-2 treatment of a patient with WAS enhanced the cytotoxicity of their NK cells and the F-actin content at the immunological synapses formed by their NK cells. IL-2 stimulation of NK cells in vitro activated the WASp homolog WAVE2, which was required for inducing WASp-independent NK cell function, but not for baseline activity. Thus, WAVE2 and WASp define parallel pathways to F-actin reorganization and function in human NK cells; although WAVE2 was not required for NK cell innate function, it was accessible through adaptive immunity via IL-2. These results demonstrate how overlapping cytoskeletal activities can utilize immunologically distinct pathways to achieve synonymous immune function.

Introduction

Wiskott-Aldrich syndrome (WAS) is an X-linked congenital human immunodeficiency characterized by the triad of susceptibility to infection, bleeding, and dermatitis. The gene mutated in WAS encodes the WAS protein (WASp), which is preferentially expressed in hematopoietic cells and facilitates reorganization of the actin cytoskeleton. The WASp C terminus contains binding domains for monomeric (G) actin as well as the actin branching complex Arp2/3. WASP binds G-actin via a verprolin homology (V) region and Arp2/3 via an acidic (A) region. When Arp2/3 and G-actin are approximated by WASp, the complex can enable formation of a branch point on an existing actin filament. The branching of filamentous actin (F-actin) allows cells to reorganize their cortex to promote subcellular and cellular motility as well as signaling needed for function. WASp serves as a critical regulator of F-actin reorganization in that it exists in an autoinhibited confirmation that prevents its ability to facilitate Arp2/3 function. After cellular activation, however, the conformation of WASp is altered to allow the approximation of Arp2/3 and G-actin at an existing actin filament, thereby enabling actin branching function.

WASp shares actin branching function with a family of proteins. Many have structural similarity to WASp and contain a C-terminal V and A region. While WASp is preferentially expressed in hematopoietic cells, other family members have critical functions in immune cells. An important example is the WASp family member 2, also known as WASp family verprolin-homologous 2 (WAVE2). A number of studies have defined critical roles for WAVE2 in T cells, in which it is required for F-actin reorganization and access to cell function (1, 2). Although WASp can support actin reorganization in T cells (3, 4), WAVE2 may be the critical facilitator. Patients with WAS, however, have been defined as having a number of T cell abnormalities including abnormal cell surface ruffling (5) as well as a decreased ability to proliferate and produce IL-2 (6–9).

Despite the existence of multiple WASp family members, the clinical immunodeficiency in WAS is wide ranging and is associated with diverse immune abnormalities. Unusual features of the disease include susceptibility to clinically relevant infections with herpesviruses (including severe cases) in approximately one-third of patients as well as hematologic malignancy representing a leading cause of death (10). This suggests a potential insufficiency in NK cell functions, since they are important for human defense against herpesviruses (11, 12) and surveillance for malignancy (13). NK cells are best known for their ability to mediate cytotoxicity after ligation of germline-encoded activating receptors. This requires the establishment of a contact between the NK cell and its target followed by the directed secretion of cytolytic molecules contained in specialized organelles called lytic granules. The contact between the NK cell and susceptible target cell is a specialized form of the immunological synapse (IS) known as the NK cell lytic IS, which progresses through a number of individual subcellular steps to facilitate cytotoxicity (14). A relatively early step in the formation of the NK cell lytic IS is the synaptic accumulation of F-actin, which is required for many subsequent steps including clustering of certain cell-surface receptors at and polarization of lytic granules to the IS (15). In human NK cells, WASp accumulates and is required for effective actin reorganization at the synapse (16).
Resting NK cells from patients with WAS are deficient in NK cell cytotoxicity and do not form an appropriate IS (15–17). In particular, F-actin accumulation at, and lytic granule polarization to, the IS are decreased. The defect in cytotoxicity is present in NK cells from patients who have a WASP mutation leading to absent protein expression as well as from patients with milder disease caused by mutations leading to decreased WASp expression (17). Even these more mildly affected patients, however, have clinical susceptibility to hematologic malignancy (18), thus furthering consideration of a linkage from the clinical phenotype to a deficiency of NK cell function.

Interestingly, the defect in WAS patient NK cell cytotoxicity is only found when cells are tested directly ex vivo and is not present after in vitro culture (16, 17). IL-2, which is present in many NK cell culture systems, can restore NK cell cytotoxicity against K562 target cells when added in vitro to WAS patient PBMCs (17). This enhancing effect was found in WAS patients having either severe or mild WASP mutations and has been defined in multiple unrelated individuals (17). Since T cells from WAS patients have a decreased ability to produce IL-2 after specific types of stimulation (6, 7, 19), it is plausible that WAS NK cells do not have access to sufficient IL-2 in vivo and cannot therefore take advantage of this effect. In the present work, we sought to define the mechanism of how IL-2 might circumvent WASP as well as determine whether there are parallel pathways to actin reorganization that can be differentially accessed in NK cells. We also attempted to determine whether this in vitro phenomenon has relevance in vivo in WAS and designed a clinical trial of IL-2 administration. The goal of this latter effort is ultimately to increase NK cell function to provide additional surveillance against malignancy and defense against herpesviruses. We report that IL-2 can directly induce F-actin reorganization in NK cells and can circumvent WASP by activating WAVE2. We also show that IL-2 can increase NK cell function and F-actin content in vivo in WAS.

Results
IL-2 induces F-actin content in NK cells independently of target cells. Short-term exposure of NK cells from WAS patients to IL-2 in vitro restores cytotoxicity and enables F-actin accumulation at the lytic IS (17). This suggested that IL-2 could provide the activation signal that would be otherwise defective in resting WAS patient NK cells upon encountering a target cell. Conversely, this result could suggest that IL-2 has a direct effect upon F-actin reorganization in NK cells and can circumvent WASP by activating WAVE2. We also show that IL-2 can increase NK cell function and F-actin content in vivo in WAS.

Effect of IL-2 on NK cell F-actin reorganization and function is WASP independent. The simplest explanation for how IL-2 could be functioning to increase F-actin content in NK cells would be via an increase in WASP levels. To evaluate this possibility, YTS or ex vivo NK cells from this patient were treated with IL-2 and total cellular WASP levels evaluated by Western blot analysis. After IL-2 incubation, neither cell culture demonstrated appreciable increases in WASP levels (Supplemental Figure 2, A and B). Alternatively, IL-2 might further induce function from existing WASP. Even though the phenomenon of IL-2–induced restoration of F-actin accumulation at the lytic synapse was originally observed in WAS patient cells harboring an aberrant WASP gene (17), IL-2 could still be acting by enabling additional functional capacity from mutant WASP. In consideration of true WASP independence of IL-2, we were able to utilize ex vivo NK cells from a patient with WAS due to a complete deletion of exons 1–12 of the WASP gene and no detectable WASP (22). Although ex vivo NK cells from this patient could form contacts with target cells, they had a reduced ability to accumulate F-actin at the IS compared with that of ex vivo NK cells from a control donor (Figure 2A and Supplemental Figure 3). When the patient ex vivo NK cells were activated with IL-2, however, increased F-actin was found at the IS. This was quantified across multiple cells using a
Figure 1

IL-2 promotes intracellular F-actin content in NK cells. (A) YTS, NK92, PBMC, or ex vivo NK cells were treated with 125 U/ml of IL-2 for 30 minutes, fixed, permeabilized, and stained with phalloidin Alexa Fluor 647. Percentage of maximal fluorescence intensity of Alexa Fluor 647 is demonstrated for control (gray) and IL-2–treated (white) cells. In PBMCs, only the CD56^+CD3^– cells were selected. (B) Phalloidin MFI induced by IL-2 over that in control-treated cells according to time of in vitro IL-2 or vehicle exposure (error bars demonstrate SD; *P < 0.05 compared with control-treated cells). (C) Distribution of actin-GFP in control-treated (top) or IL-2–treated (bottom) YTS GFP-actin cells using real-time confocal microscopy. GFP-actin fluorescence is depicted in green, and DRAQ5 DNA dye (nuclear material) is depicted in red. Time = 0:00 represents the addition of IL-2 or vehicle to the imaging chamber. Scale bar: 5 μm. (D) Calculated change from time = 0:00 in mean area intensity of peripheral GFP-actin over the first 8 minutes in control-treated cells (gray triangles) compared with IL-2–treated cells (black squares). Each point represents the mean ± SD from 10 cells, and the IL-2 and control data sets are significantly different (P = 0.0023) via a 2-tailed Mann-Whitney U test. (E) Insoluble F-actin–rich cell fractions prepared from unstimulated, 10-minute, or 120-minute IL-2–stimulated YTS GFP-actin cells were evaluated by Western blot analysis for GFP-actin (top), endogenous actin (middle), and histone H1 (bottom). The numbers beneath the blots provide the densitometric ratio of the GFP or actin signal to that present in the unstimulated lane and are representative of 2 repeats.
previously validated algorithm (23). In unstimulated patient ex vivo NK cells, there was no mean synaptic F-actin accumulation, but IL-2 induced levels to those found in control cells (Figure 2B). This increase in F-actin content was also associated with maturation of the IS, as only after IL-2 stimulation was perforin able to polarize to the IS in patient ex vivo NK cells (Figure 2A), as measured by the distance of the perforin region centroid to the synapse across multiple cells (Figure 2C). Thus, even in the absence of the WASP gene, IL-2 is able to promote actin reorganization.

As an alternative to the WASP deleted cells, the small molecule inhibitor of WASp, wiskostatin, was utilized. Wiskostatin stabilizes the autoinhibited conformation of WASp, thus blocking its ability to promote actin branching (24). Wiskostatin also inhibits N-WASP, which is a ubiquitously expressed WASP homolog. While N-WASP is not able to compensate for WASp in patients with WAS, it is possible that IL-2 was inducing function in NK cells through N-WASP. In NK92 or YTS cells or in PBMCs, wiskostatin pretreatment did not reduce WASp levels (Supplemental Figure 2, A and B), but was able to inhibit the majority of cytolytic activity (Figure 3A). Addition of IL-2 to the cytotoxicity assay, however, improved function in both control and wiskostatin-treated NK92 cells or PBMCs. Control-treated YTS cells had only minimal increase in cytotoxicity after IL-2 stimulation, but IL-2 restored activity in wiskostatin-treated cells to control levels. To determine whether IL-2 could induce a target cell–independent effect in the presence of wiskostatin, inhibitor- or vehicle-treated NK92 cells, YTS cells, or PBMCs were treated with IL-2 and evaluated for F-actin content. Although wiskostatin pretreatment reduced F-actin levels (at least in NK-92 cells and PBMCs, presumably by preventing new F-actin formation during actin turnover), addition of IL-2 to wiskostatin-treated cells increased the F-actin content toward control levels (Figure 3B). Since WASp promotes actin branching, we also evaluated the ultrastructure of F-actin at the IS in

Figure 2
IL-2 restores F-actin accumulation at and lytic granule polarization to the NK cell IS. (A) Representative examples of the NK cell IS formed between resting or 30-minute IL-2–stimulated ex vivo NK cells obtained from either a control donor (control) or a WAS patient with a complete deletion of the WASP gene (WASPdel) with K562 target cells (additional examples in Supplemental Figure 3). Images show DIC (top panel) and confocal fluorescence microscopy for F-actin using phalloidin (yellow) and perforin (green) as well as an overlay of fluorescent channels. Scale bars: 5 μm. (B) Quantification of F-actin accumulation at the IS formed between K562 target cells and ex vivo NK cells of control (red), IL-2–activated control (orange), WASPdel patient (black), and IL-2–activated WASPdel patient (blue). Each spot represents the calculated F-actin accumulation value (see Methods for details) from a single cell; the horizontal bar represents the mean of all cells shown and the vertical bars ± SD. Significant differences among group means are shown. *P < 0.05; n ≥ 25. (C) Measurement of lytic granule polarization as the shortest distance from the centroid of the entire lytic granule region to the IS in each of the ex vivo NK cell–target cell combinations. Bar colors correspond to those used in B and display mean ± SD. Mean distance in resting WASPdel patient ex vivo NK cells was different from that in the other cells measured. *P < 0.05; n ≥ 25.
IL-2 increases cytotoxicity and F-actin reorganization in wiskostatin-treated NK cells. (A) Cytotoxic activity of YTS cells (left), NK92 cells (middle), or PBMCs (right) pretreated with vehicle control (c, black solid line), IL-2 (gray solid line), wiskostatin (Wisk, black dashed line), and wiskostatin followed by IL-2 (Wisk+IL-2, gray dashed line). Cells were incubated with IL-2 where specified for 30 minutes prior to the assay. K562 target cells were used for NK92, and 721.221 target cells for YTS and PBMCs. Cytotoxicity was measured by 4-hour $^{51}$Cr-release assays. Each point represents the mean of 5 individual assays, each performed in triplicate ± SD. For each graph, decreases mediated by wiskostatin and increases in wiskostatin-treated cells mediated by IL-2 were significant (*$P < 0.05$, 2-tailed Mann-Whitney U test). (B) Change in F-actin content measured as percentage increase in phalloidin MFI via flow cytometry in YTS cells (left), NK92 cells (middle), or PBMCs (right). Vehicle control-treated cells were compared with IL-2–treated (black), wiskostatin-treated (gray), and wiskostatin- and IL-2–treated (white). Each bar represents the mean of 3 or more experiments ± SD (*$P < 0.05$, significant changes from control-treated cells). 30-minute wiskostatin pretreatment was followed by 30-minute IL-2 treatment. (C) Representative TEM images of platinum rotary-shadowed, sonicated, and extracted YTS cells that had been adhered to glass coated with CD18 and anti-CD28. Prior to adherence to the glass, cells were treated with vehicle (left), wiskostatin (center), or wiskostatin followed by IL-2 (right). Cortical F-actin network detail is shown. Original magnification, ×6000 (scale bar: 5 μm [top]) and ×30,000 (scale bar: 1 μm [bottom]).
activated NK cells using platinum replica electron microscopy. YTS cells were adhered to glass coated with anti-CD18 and anti-CD28 to promote adhesion and activation and then extracted to remove all but the cell cortex. In control-treated activated cells, a branched actin network was evident, which was diminished when cells were treated with wiskostatin prior to activation (Fig. 3C). When the wiskostatin-pretreated cells were treated with IL-2 during activation, however, a branched actin network was restored, albeit perhaps qualitatively distinct from control-treated cells (solid line). The control cytotoxicity for all of the treatments is in the left-most graph (black circles). Cytotoxic activity of treated cells was measured against 721 or K562 target cells in 4-hour 51Cr-release assays. Individual points represent the mean of triplicates, and individual experiments are representative of 2–5 repeats. (B, D, F) A portion of the cells prepared for cytotoxicity was used to prepare whole-cell lysates, which were evaluated for the presence of phosphorylated (top) and total (bottom) STAT5 by Western blot analysis. Total STAT5 was detected using the same membrane used for phosphorylated STAT5 after stripping and reprobing. Numbers beneath each lane represent densitometric ratios for pSTAT5 to total STAT5.

Figure 4
Requirement for IL-2 signaling to rescue NK cell cytotoxicity from WASp inhibition. YTS (A and B), NK92 (C and D), and ex vivo NK (E and F) cells were control (C, circles), wiskostatin (Wisk, squares), ZM 449829 (downward triangles), or wiskostatin and ZM 449829 (Wisk-ZM, upward triangles) treated prior to addition of media (solid black line) or IL-2 (dashed gray line) for 30 minutes. Each column of graphs depicts the effect of IL-2 (dashed line) added to the respective control or inhibitor-treated cells (solid line). The control cytotoxicity for all of the treatments is in the left-most graph (black circles). Cytotoxic activity of treated cells was measured against 721 or K562 target cells in 4-hour 51Cr-release assays. Individual points represent the mean of triplicates, and individual experiments are representative of 2–5 repeats. (B, D, F) A portion of the cells prepared for cytotoxicity was used to prepare whole-cell lysates, which were evaluated for the presence of phosphorylated (top) and total (bottom) STAT5 by Western blot analysis. Total STAT5 was detected using the same membrane used for phosphorylated STAT5 after stripping and reprobing. Numbers beneath each lane represent densitometric ratios for pSTAT5 to total STAT5.

We next wanted to determine whether the WASp-independent effect of IL-2 in promoting NK cell function was dependent upon classical IL-2 signaling. Thus, we utilized the JAK3 inhibitor ZM449829 (25) to interfere with IL-2 signal transduction. While YTS, NK92, or ex vivo NK cells pretreated for 30 minutes with ZM449829 were not inhibited in cytotoxicity, IL-2 was no longer able to rescue cytotoxicity after wiskostatin pretreatment (Figure 4, A, C, and E). To define the IL-2 signal in the presence of ZM449829 and/or wiskostatin, STAT5 phosphorylation was evaluated by Western blot analysis. IL-2 treatment of YTS, NK92, or ex vivo NK cells robustly induced STAT5 phosphorylation. Wiskostatin pretreatment did not interfere, but ZM449829 blocked IL-2–induced STAT5 phosphorylation (Figure 4, B, D, and F). Thus, while wiskostatin does not appear to interfere with IL-2 signal generation, IL-2 signaling was required for the ability of IL-2 to circumvent inhibition of WASp in NK cells.

In vivo impact of IL-2 upon NK cells in WAS. Since WAS is associated with a potentially clinically relevant deficiency of NK cell function, we hypothesized that in vivo IL-2 administration to patients might induce the WASp-independent NK cell effects observed in vitro. Therefore, a clinical trial of IL-2 administration in WAS was organized, and a patient with a 134C>T WASP mutation was treated with 0.5 × 10^6 U of IL-2/m^2 subcutaneously for 5 days. Treatment was repeated twice at 2-month intervals (Figure 5A). The patient tolerated IL-2 injections and experienced local reactions (Figure 5B), but otherwise finished the protocol uneventfully. A variety of assessments were performed in consideration of the impact of in vivo IL-2 administration, including NK cell cytotoxicity against K562 target cells measured among ex vivo PBMCs. As expected, the patient had decreased NK cell cytotoxicity pretreatment compared...
with control, with values similar to other patients having analogous WASP mutations (16, 17). After IL-2 administration (week 23), however, cytotoxicity was similar to control (Figure 5C). NK cell cytotoxicity was measured in samples obtained throughout the protocol and expressed as lytic units (LU$_{20}$, lytic units at 20% lysis) per NK cell. While there was not an increase in response immediately following all 3 courses of IL-2, after the second and third, the baseline LU$_{20}$ remained elevated above the level present prior to starting the protocol (Supplemental Figure 4A). This suggests some lasting impact of the IL-2, even though pharmacokinetic assessments of similar doses in other populations would predict that circulating administered IL-2 would no longer be apparent (26). The elevated baseline lytic units at the end of the protocol were not reflected by shifts in the NK cell population, as the percentage of NK cells or distribution of CD56$^\text{dim}$ and CD56$^\text{bright}$ NK cells was not consistently altered over the treatment period (Supplemental Figure 4, B and C). There were also no major consistent shifts in the percentages of total CD3$^+$ T cells, although individual subpopulations were not evaluated (Supplemental Figure 4D).

To evaluate the effect of in vivo IL-2 upon NK cell F-actin content, ex vivo patient cells were studied using fluorescent phalloidin. WAS patient CD56$^\text{dim}$ or CD56$^\text{bright}$ NK cells had lower pretreatment phalloidin MFI compared with control (Figure 5D). After the first and second courses of IL-2 administration, increases in F-actin content in both CD56$^\text{dim}$ and CD56$^\text{bright}$ NK cells were observed relative to control. There was an accentuated effect in the CD56$^\text{bright}$ population, which may be a feature of high-affinity IL-2 receptor, which is preferentially expressed on this subpopulation (27). To directly measure F-actin in NK cells from the patient in a functionally relevant context, the lytic IS formed with K562 target cells ex vivo was evaluated. Accumulation of F-actin and polarization of perforin were identified at the IS after 30 minutes in control donor, but not pretreatment WAS patient, cells (Figure 6, A and B). Patient samples analyzed after each of the 3 courses of IL-2, however, demonstrated a restored lytic synapse with F-actin accumulation and perforin polarization (Figure 6, A–C). In all 3 posttreatment patient samples, synaptic F-actin content or perforin polarization was significantly improved compared with pretreatment and was similar to control donor values. These data demonstrate a functional impact of IL-2 on the NK cell population in vivo in a patient with WAS and further suggest the access of IL-2 to a WASp-independent pathway for NK cell function.

WASp-independent activity of IL-2 requires WAVE2. We next wanted to determine how IL-2 was accessing NK cell actin reorganization and function independently of WASp. Although NK cells rely heavily upon WASp as an Arp2/3 activator for F-actin branching, the major protein serving this function in T cells is WAVE2 (1).
Thus, we attempted to identify WAVE2 in NK cells and to determine whether levels were induced by IL-2 stimulation. WAVE2 was present in both YTS and ex vivo NK cells, but levels were not increased after 30 minutes of IL-2 stimulation (Figure 7, A and B). To determine whether WASp inhibition might uncover an ability of WAVE2 to be upregulated by IL-2, cells were pretreated with wiskostatin prior to stimulation. While wiskostatin pretreatment did not alter WAVE2 levels, they were not further increased by IL-2. It was still possible, however, that IL-2 was activating WAVE2 in NK cells. WAVE2 activation requires Rac activation, acidic phospholipids, and WAVE2 serine phosphorylation (28). To determine whether the signal for WAVE2 activation was induced in NK cells after IL-2 stimulation, we evaluated the presence of active Rac using a GTP-Rac pull-down assay. GTP-Rac was increased more than 3-fold after 30 minutes of IL-2 exposure and was not negatively affected by preincubation with wiskostatin (Supplemental Figure 5). This suggests that WAVE2 activation can occur after IL-2 stimulation and independently of WASp function. Because WAVE2 is phosphorylated upon activation (29) and specific serine phosphorylation is required for WAVE2 function (28), we next evaluated WAVE2 for serine phosphorylation following IL-2 stimulation in NK cells. YTS cells were pretreated with vehicle or wiskostatin and treated with IL-2 for 30 minutes; WAVE2 was isolated via immunoprecipitation. Phosphorylated STAT5 was measured in the supernatant to determine whether cells had received an IL-2 signal, and as expected, was only present after IL-2 exposure and was unaffected by wiskostatin (Figure 7C). Also as expected, WAVE2 was present in the immunoprecipitate (Figure 7D).

IL-2 stimulation induced phosphoserine signal at the WAVE2 band (Figure 7, D and E). Although wiskostatin treatment decreased baseline phosphoserine levels at the WAVE2 band, they were induced approximately 8-fold by IL-2 stimulation in wiskostatin-treated cells. This experiment was also performed using ex vivo NK cells. WAVE2 was present in the ex vivo NK cell immunoprecipitate, and IL-2 induced serine phosphorylation at the WAVE2 band (Figure 7, F and G). Similar to YTS cells, the baseline phosphoserine in the WAVE2 band was decreased by wiskostatin pretreatment, but was increased approximately 6-fold by IL-2. To confirm this finding in the absence of WASp, the WAVE2 immunoprecipitate from NK cells from the patient with a deleted WASp gene (Figure 2) was evaluated. IL-2 induced

Figure 6
In vivo IL-2 administration of IL-2 to a WAS patient restores F-actin accumulation at and lytic granule polarization to the lytic IS in NK cells. (A) Representative images (from left to right) of the NK cell IS formed between an ex vivo NK cell from a control donor or the WAS patient prior to or after each of the 3 courses of IL-2 treatment and K562 target cell. A DIC image (top panel) and confocal fluorescence micrographs (bottom panels) depicting F-actin (phalloidin, yellow) and perforin (green) as well as an overlay of fluorescent channels. Scale bars: 5 μm. Quantitation of F-actin accumulation at (B) and polarization of the lytic granule region to the IS (C) in control donor (red) and patient before (black) as well as after the first (light green), second (dark green), and third (brown) IL-2 treatments as presented in Figure 2. Each mean represents 15 synapses ± SD and significant differences are noted. *P < 0.05.
phosphoserine levels at the WAVE2 band in both control and patient NK cells (Figure 7, H and I). Thus, IL-2 activated WAVE2 in NK cells as determined by phosphorylation, even in the presence of WASp inhibition or in the absence of WASp.

To determine whether WAVE2 was required for the WASp-independent effect of IL-2 upon NK cell function, we established WAVE2 siRNA in YTS and ex vivo NK cells. Compared with control siRNA, WAVE2-specific siRNA reduced WAVE2 levels by 70%–80% (Figure 8, A and B). Cells treated with siRNA were then further treated with wiskostatin and/or IL-2 and evaluated for cytotoxicity. In control siRNA–treated cells, wiskostatin inhibited killing and IL-2 was able to restore cytotoxicity to baseline levels (Figure 8, C and D). WAVE2 siRNA negligibly affected baseline cytotoxic activity, but completely inhibited the ability of IL-2 to restore cytotoxicity after wiskostatin treatment (Figure 8, C and D). Thus, WAVE2 was required for WASp-independent access to cytotoxicity. To determine whether this was reflected at the level of F-actin, the target cell–independent ability of IL-2 to increase F-actin content and partially compensate for WASp inhibition was evaluated. In control siRNA–treated cells, IL-2 increased F-actin content and IL-2 was still able to increase F-actin content in wiskostatin-treated cells (Figure 8, E and F). In WAVE2 siRNA–treated cells, however, the ability of IL-2 to increase F-actin content in wiskostatin-treated cells was lost. To confirm that IL-2 signaling was still active in these cells and had not been perturbed by reduced WAVE2 expression, STAT5 phosphorylation levels were determined in YTS cells. In all cases, IL-2 was still able to robustly induce STAT5 phosphorylation (Figure 8G), demonstrating that the role of WAVE2 is downstream of the IL-2 signal. Thus, our results demonstrate the existence of a WASp-independent pathway for actin reorganization accessible by IL-2 and dependent upon WAVE2.

Discussion

NK cells depend upon actin reorganization to effectively form and access cytolytic function through the IS. A central role for WASp in this process has been defined through studies of ex vivo NK cells from patients with WAS (11, 15, 17). Patient cells are defective in cytotoxicity and have a reduced ability to accumulate herpesviruses and hematologic malignancies, it is feasible that their deficiency of NK cell function is clinically relevant. The identification of deficient NK cell function in WAS presented a challenge, however, because IL-2 as a standard additive to human NK cell culture restores NK cell cytotoxicity. In the present work, we defined the mechanism of this IL-2–mediated effect and therein identified a second pathway for actin organization in NK cells. We also applied it to WAS to determine proof of principle in consideration of clinical application.

Unexpectedly, we found IL-2 had a direct impact upon F-actin in NK cells. When provided in solution, it increased F-actin content independently of the presence of target cells. Thus, IL-2 would be
expected to promote F-actin reorganization diffusely in NK cells since it would derive signal from abundant cell-surface IL-2 receptors (27). In support, diffuse real-time reorganization after IL-2 exposure was identified in GFP-actin YTS cells (Figure 1). The effect of IL-2 upon F-actin in suspension culture NK cells did not require WASp, as IL-2 was able to circumvent blockade resulting from the WASp-inhibitor wiskostatin. This demonstrated that a cell contact–independent stimulus could induce a distinct means of promoting F-actin organization.

F-actin induction by IL-2 also could occur independently of WASp when a cell contact was present. This was demonstrated using wiskostatin and patient cells completely lacking the WASp gene by increases in, or relocalization of, phalloidin staining as well as the ultrastructure of actin filaments. NK cell cytotoxicity, which requires actin reorganization, followed suit. The effect of IL-2 upon F-actin was unlikely to be via the induction of the WASp homolog N-WASp, since wiskostatin was originally defined for its ability to inhibit N-WASp (24). A distinct Arp2/3 activator was therefore pursued.
IL-2 directly induced phosphorylation of the Arp2/3 activator WAVE2. This could occur independently of stimulatory inputs derived from the lytic IS, as it was identified in NK cells in suspension (Figure 7). Although the regulation of WAVE2 is the subject of ongoing investigation, phosphorylation of the protein is required for WAVE2-mediated actin nucleation (28). Known signals inducing WAVE2 phosphorylation include serum starvation (28, 29), PDGF (29), and EGF (28), but not IL-2. In these systems, acidic phospholipids and Rac activation are also required for WAVE2 activation. In this light, we found Rac to be activated after IL-2 stimulation and to occur independently of WASp function (Supplemental Figure 5). The same was true for WAVE2 phosphorylation (Figure 7, D–I). Sufficient numbers of NK cells, however, were only available to perform these experiments using healthy donor cells treated with wiskostatin in vitro or cells from the WASp-deleted patient but not the patient treated with IL-2 in vivo. Regardless, since the WASp-independent restoration of cytotoxicity was dependent upon IL-2 signaling (Figure 3), it would appear that IL-2 has specific WASp-independent access to WAVE2 activation. Importantly, WAVE2 was required for the ability of IL-2 to rescue NK cells from WASp blockade, as demonstrated using siRNA. WAVE2, however, was not required for the majority of baseline cytolytic function in NK cells. Thus, IL-2 represents what we believe to be a novel WAVE2 activator and an alternative mechanism to that directed by WASp for accessing F-actin reorganization in NK cells.

The utility of WAVE2 suggests 2 distinct pathways to F-actin reorganization in NK cells. The first is dependent on WASp and presumably accessible through routine contact-dependent activation signals, such as those provided through lytic triggering receptors. This is likely to be the default pathway in NK cells, since they do not produce significant IL-2. This requirement is further suggested by the NK cell deficiency identified in WAS patients (16, 17). The second pathway uses WAVE2 and is inducible and accessible in NK cells through a soluble IL-2 signal. Once induced, however, this pathway can enable the directed accumulation of F-actin at the IS and likely permits WAVE2 to substitute for WASp at the IS downstream of activating receptors. It is presently unclear if the requirement for IL-2 is in targeting WAVE2 to the IS, or for activation of WAVE2 irrespective of subcellular localization. While a role for IL-2 has not been previously investigated, the WAVE2 pathway has been shown to be the default in T cells and required for function downstream of the TCR (1, 2, 30, 31). The role for WASp in T cells regarding TCR-induced actin accumulation is less clear (3, 4, 32). It is possible, however, that some tonic exposure to IL-2 in the context of routine T cell expansion may constitutively enable the WAVE2 pathway in mature T cells. This pathway may be defective in WAS due to the requirement for WASp in production of IL-2 by human T cells. An IL-2 production defect in WAS patient T cells has been demonstrated after in vitro activation of cells using certain stimuli, including mitogens or immobilized anti-TCR antibodies (6–9) as well as some (19), but not all, (7) antigen-presenting cell systems. Decreased IL-2 production by WASp-deficient T cells after stimulation has also been defined in mice (3, 4, 33).

This dichotomy establishes a difficult impasse in WAS, one in which NK cells can utilize neither the default WASp pathway for genetic reasons nor the WAVE2 pathway fully due to potentially inadequate IL-2 supply from T cells. Given that patients are affected by clinical conditions associated with defective NK cell defense, this suggests the opportunity to intervene by enabling the WASp-independent pathway therapeutically using IL-2. IL-2 had been previously administered with reported clinical benefit to a WAS patient who had intractable HSV infection, but detailed immune evaluations including those of NK cells were not performed (34). In the present work we were able to study the impact of IL-2 administration upon NK cells in a WAS patient. Although IL-2 therapy is not benign and has many associated adverse effects, the dose used in the patient treated was well tolerated, substantially lower than those typically associated with severe toxicities and similar to those in other studies not associated with severe toxicity (35). This may suggest an opportunity, however, to consider other potentially better tolerated therapeutic cytokines using the common γ-chain for signaling. In this light, we have preliminarily found that IL-15 can also induce actin content in NK cells and differences between the common γ-chain utilizing cytokines need to be considered in detail.

Our initial experience with in vivo IL-2 administration, however, demonstrated at least some degree of enhanced NK cell cytotoxicity, F-actin content, and lytic IS formation. While further study is indicated, these initial results also suggest some sustained impact of IL-2 on NK cells past what might be expected given the pharmacokinetics of IL-2 (26). This could potentially be explained by some restorative effect of IL-2 on patient T cells (as has been described in vitro; refs. 19, 36), perhaps enabling them to produce IL-2 and drive the WAVE2 pathway in NK cells, and represents a mechanism warranting future investigation. Importantly, however, as NK cell deficiency is present in even mild WAS patients (17) for whom hematopoietic stem cell therapies might not represent an acceptable risk, IL-2 may present a reasonable option. A majority of these more mildly affected patients live past childhood, but have a median event-free survival of 10 years with a 5% risk of lymphoid malignancy (18). Thus, intermittent application of IL-2 might facilitate NK cell surveillance functions to improve outcome in these more mildly affected patients and will be a focus of future work.

While the ability to access the WAVE2 pathway in NK cells therapeutically is potentially clinically relevant in WAS, it also provides insight into mechanisms underlying cooperation between adaptive and innate immunity. Specifically, T cells could provide IL-2 to enable enhanced WAVE2-dependent F-actin organization in NK cells upon their encountering a susceptible target. This defines a mechanism of how T cells may augment innate NK cell functions during an immune response. Since this can be accomplished with soluble IL-2, it could presumably be exploited by local or even distantly activated T cells. This presents a new potential mechanism for how adaptive immunity could cooperate with innate function in enhancing the response against host cells that may have become damaged, but do not present the specific antigen that would be recognized by responding T cells. Thus, dual pathways to actin reorganization can be differentially accessed and likely serve distinct functions in the immune response.

**Methods**

**NK cells, cell lines, and in vitro treatments.** The YTS and NK-92 immortalized NK cell lines, both derived from malignant expansions of NK cells, as well as the target cell K562 erythroleukemia line and 721.221 lymphoblastoid line were grown and maintained in culture as described previously (37). PBMCs were isolated from healthy volunteers using Ficoll Hypaque (Amersham) from which NK cells were isolated by negative selection where necessary. PBMCs were isolated from healthy volunteers using Ficoll Hypaque (Amersham) from which NK cells were isolated by negative selection where necessary. PBMCs were isolated from healthy volunteers using Ficoll Hypaque (Amersham) from which NK cells were isolated by negative selection where necessary. PBMCs were isolated from healthy volunteers using Ficoll Hypaque (Amersham) from which NK cells were isolated by negative selection where necessary.

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cells. PBMCs or ex vivo NK cells were used for experiments immediately after isolation. Splenocytes from a previously defined patient with WAS due to complete deletion of exons 1–12 of WASp (22) were used from cryo-preserved samples where indicated for preparation of NK cells.

Where specified, cells were treated in vitro with 3% w/v sodium deoxycholate (Cal-Biochem) or 1 μM ZM449829 (Tocris Bioscience) in the RPMI-based complete media containing 10% FCS for 30 minutes at 37°C. The inhibitor stock solutions contained DMSO, and thus an equivalent amount of DMSO in media was used as a vehicle control. Optimal inhibitor concentrations were derived from dose-citration experiments using viability, cytotoxicity, and STAT5 phosphorylation (ZM449829 only) as end points (data not shown). For wiskostatin, the minimal concentration inhibiting cytotoxicity was pursued, whereas for ZM449829, the minimal concentration inhibiting STAT5 phosphorylation was targeted. IL-2 (Novartis) at 125 U/ml in media was used for cell stimulation unless otherwise stated. The concentration was derived from dose-response experiments evaluating cytotoxicity and F-actin content. For measurement of Rac1 activation, 24-hour–serum-starved YTS cells were treated with vehicle or wiskostatin with or without IL-2 prior to cell lysis. When IL-2 was used in inhibitor-treated cells, IL-2 was always added for the specified time after the 30-minute inhibitor pretreatment.

Cytotoxicity. Four-hour 31Cr-release assays were performed in round-bottom 96-well microtiter plates as described (38). YTS, PBMCs, and ex vivo NK cells were used as effectors against 721.221 target cells or NK92 cells as effectors against K562 target cells. The highest effector-to-target cell ratios (E:T) used were 10:1 for YTS and NK92 cells, 20:1 for ex vivo NK cells, and 50:1 for PBMCs. LU20 and LU50 per NK cell were calculated as previously described (16).

Flow cytometry. Flow cytometry was performed for the routine identification of cell subsets among PBMCs and to evaluate purified ex vivo NK cells as described (16). NK cells were defined as CD56 CD3– and T cells as CD3+ CD56–. The CD56 bright and CD56 dim NK cells were defined by a gate applied to the nadir between the 2 populations in a histogram analysis of CD56 fluorescence in CD3– cells. Flow cytometry for F-actin content was performed by first incubating cells with antibodies against cell-surface markers for 15 minutes to allow for NK cell identification, followed by permeabilization and fixation using Cytofix/Cytoperm solution (BD Biosciences). Fixed cells were washed 3 times with Perm/Wash solution (BD), incubated for 30 minutes in 4°C with Alexa Fluor 647–conjugated phalloidin (Invitrogen), washed 3 times with Perm/Wash, and then fixed in 2% paraformaldehyde. The percentage change in the MFI in Alexa Fluor 647 phalloidin levels was calculated as follows: ([MFI_{experimental} − MFI_{control}]/MFI_{control}) × 100.

Immunoprecipitation and Western blot analysis. For immunoprecipitation, cells were lysed in 25 mM Tris-Cl (pH 7.5) containing 150 mM NaCl, 5 mM MgCl2, 1% NP-40, 1 mM DTT, 5% glycerol with protease, and phosphatase inhibitor cocktail (Roche) on ice for 30 minutes. For Western blot analysis of cells only, lysis was performed directly in NuPAGE LDS sample buffer (Invitrogen), directly followed by boiling for 5 minutes prior to gel loading. Proteins were separated on 4–12% NuPAGE Bis-Tris gels in MOPS SDS running buffer and transferred onto nitrocellulose membranes (Invitrogen). The membranes were blocked in 3% BSA and incubated with an antibody directed against 1 of the following proteins for 1 hour: WASp (clone Sa5; BD), myosin-IIA (polyclonal M8064; Sigma-Aldrich), Rac-1 (monoclonal ARCO2; Cytoskeleton), STAT5 (polyclonal C-17; Santa Cruz Biotechnology Inc.), phosphoSTAT5 Y694 (clone E208; Abcam), WAVE2 (polyclonal H-110; Santa Cruz Biotechnology Inc.), or phosphoserine (polyclonal Z-PS1; Invitrogen). The membranes were washed, then incubated with secondary antibody for 1 hour, and washed again; bound antibody was detected using ECLplus (Amersham). Where specified, membranes were stripped using 0.2 M glycine (pH 2.5, 0.05% Tween-20, and 140 mM NaCl in TRIS-buffered saline at 50°C for 30 minutes prior to blocking and reprobing to detect a different protein. Band intensities were measured using Image J software (NIH Shareware).

For immunoprecipitation, lysates were centrifuged at 14,000 g and the supernatant preclarred with protein G agarose (Invitrogen) for 30 minutes at 4°C and centrifuged again to remove beads. Cleared supernatant was then incubated at 4°C for 1 hour with protein G agarose that had been incubated overnight with 2 μg antibody, centrifuged, washed 3 times in lysis buffer, and centrifuged to separate supernatant and precipitate. Activated Rac1 was precipitated from cell lysates using PAK-1 p21 binding domain GST fusion protein agarose beads (Cytoskeleton).

Cytoskeletal fractionation and analysis of F-actin content. Isolation of insoluble F-actin–rich fractions from cell lysates was performed as described (39). Briefly, 1 × 105 YTS cells expressing GFP-actin were either left unstimulated in R-10 media or stimulated by the addition of 125 U/ml IL-2. Cells were collected by centrifugation, lysed in cytoskeletal stabilizing lysis buffer (80 mM PIPES [pH 6.9], 1% Triton X-100, 1 mM EGTA, 1 mM MgCl2, HALT protease inhibitor cocktail [Pierce]), and centrifuged at 5000 g for 5 minutes. The F-actin–rich pellet, also containing nuclei, was resuspended in high-salt buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, HALT protease inhibitor cocktail) and vortexed for 1 hour at 4°C to extract proteins. Samples were subjected to centrifugation at 16,000 g for 10 minutes, and supernatants representing 2 × 105 cell equivalents were used for Western blot analysis with anti-actin (polyclonal A5060, Sigma-Aldrich) or anti-GFP antibodies (monoclonal 9996; Santa Cruz Biotechnology Inc.), thus allowing distinction of GFP-actin and endogenous actin. Endogenous and GFP-actin were additionally distinguished by molecular weight difference. In addition to analysis of GFP-actin and endogenous F-actin content, the nuclear protein histone H1 was used as a loading control (monoclonal 8030; Santa Cruz Biotechnology Inc.) for F-actin–rich fractions.

Microscopy and image analysis. Cells were prepared for evaluation of fixed conjugates by immunofluorescent microscopy as previously described (37). Live cell confocal microscopy was performed as described previously (38) but employed YTS cells stably expressing human actin fused to GFP (GFP-actin). These cells were generated via transduction of a retroviral vector (provided by Janis Burkardt, Children’s Hospital of Philadelphia) as previously reported (37). To evaluate GFP-actin redistribution, cells were loaded with 5 μM DRAQ5 (Biostatus LTD) for 5 minutes at room temperature to allow nuclear visualization, washed, and added to an environmentally controlled imaging chamber (Biopaths AT system) in 0.5 ml media; after cells were stably visualized in the field of view, IL-2 or vehicle was added to the chamber media. TIRF microscopy was performed as described (38), but using YTS-GFP or YTS–GFP-actin cells adhered to glass coverslips precoated with anti-CD18 (clone TS1.18.1; ATCC) and anti-NKp30 (clone Z25; Beckman Coulter). These were stained with phalloidin prior to fixation on the slides as per the fixed cell conjugates. Microscopy was performed using a spinning-disc confocal, TIRF microscope (Olympus IX81 with DSU) employing a x60 1.49NA objective and a Hamamatsu EM-CCD camera. Illumination was provided for confocal microscopy using an Olympus 100 W halogen source with narrow bandpass filters (Chroma) and for TIRF via 488- and 568-nm lasers through a LSM5 laser combiner unit (Spectral Imaging). For some experiments, a Zeiss (Axio observer Z1) fluorescence microscope was used, which was outfitted with a x63 1.42 NA objective and Yokogawa CSU-10 spinning disc with illumination provided from 405-nm, 488-nm, 565-nm, and 655-nm lasers through an LSM5 laser combiner unit (Spectral). Images were acquired using Velocity (PerkinElmer) acquisition and analyzed using the Velocity classification module. Previously described and validated algorithms were utilized to measure F-actin accumulation (23) and perform distance from the IS (37). GFP margination in live cells was measured by comparing the MFI of the cell footprint to

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For transmission electron microscopy, YTS cells were activated for 30 minutes on glass cover slips coated with α-CD28 (clone 9.3) and α-CD11a (clone TS1/22). Covers slips were rinsed in PBS, immersed for 10 seconds into 1/3x PEM buffer (0.1 M PIPES [Sigma-Aldrich], pH 6.9, 1 mM EGTA [Sigma-Aldrich], 1 mM MgCl₂ [Sigma-Aldrich]), sonicated in PEM, incubated for 1–2 minutes in 1% Triton X-100 in PEM, and fixed in 2% glutaraldehyde in PEM. Platinum replicas were made as described (40) and imaged on a JEM 1011 transmission electron microscope (JEOL USA) with an ORIUS 835.10W CCD camera (Gatan).

In vivo administration of IL-2. The protocol for administration of IL-2 (ClinicalTrials.gov identifier NCT00774358) was approved by the Internal Review Board for the Protection of Human Subjects of the Children’s Hospital of Philadelphia (protocol number 2007-6-5354) and is in accordance with an investigational new drug approval (#101559) from the US Food and Drug Administration for a phase I clinical trial. While this particular trial is ongoing, the experience from the first enrollment is presented for proof of concept. A 13-year-old patient with a 134C>T WASP mutation predicting a T45M missense alteration previously associated with 80% reduction in WASP levels (22) was enrolled in the protocol after assent and parental consent. The patient had grade-2 WAS according to the WAS severity scale (41) and had persistent thrombocytopenia, recurrent respiratory tract infection, and a family history of maternal uncles who died prematurely from hematologic malignancy. After screening, IL-2 (Novartis) was administered subcutaneously at 1.4 U/m². IL-2 was imaged on a JEM 1011 transmission electron microscope (JEOL USA) with a 140.05W CCD camera (Gatan).

Statistics. Data were compared using Student’s 2-tailed t test or 2-tailed Mann-Whitney U test (where specified), and significance defined as P < 0.05.

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