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The Polarization Defect of Wiskott-Aldrich Syndrome Macrophages Is Linked to Dislocalization of the Arp2/3 Complex

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Wiskott-Aldrich syndrome (WAS) patients suffer from recurrent infections with recurrent bacterial and viral infections, indicating a profound immune cell defect. Such altered immune cells include monocytes, macrophages, and dendritic cells, which were reported to display disturbed cell polarization or chemotaxis. WAS is caused by mutations in the WAS protein (WASp), which is thought to organize the actin cytoskeleton through the Arp2/3 complex. Here we show that the Arp2/3 complex is an integral part of podosomes, actin-rich adhesion structures of macrophages, and that WAS macrophages fail to organize the Arp2/3 complex into podosomes. We also demonstrate that microinjection of a C-terminal acidic stretch of WASp into normal macrophages displaces Arp2/3 from podosomes and, in combination with chemoattractant stimulation of cells, induces a phenotype resembling the polarization-defective phenotype of stimulated WAS macrophages. These findings point to an important role of the Arp2/3 complex in polarization and migration of immune cells. 

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4 Abbreviations used in this paper: WAS, Wiskott-Aldrich syndrome; WASp, WAS protein.
Cells were identified by coinjected, lysine-fixable FITC dextran (100 m; Molecular Probes). Control injections were performed with GST.

The phenotypes of single polarized cells gained with both methods were and fully sequenced. Proteins were expressed in Piscataway, NJ). Inserts of constructs were checked for correct orientation BamHI site (in the case of construct VC) of vector pGEX-2T (Pharmacia, Piscataway, NJ). Inserts of constructs were checked for correct orientation and fully sequenced. Proteins were expressed in Escherichia coli as GST fusions as described earlier (18). Purity was tested by SDS-PAGE and Coomassie staining.

Immunofluorescence microscopy
Cells were fixed for 10 min in 3.7% formaldehyde solution and permeabilized for 10 min in ice-cold acetone. Samples were processed as described previously (17). Briefly, actin was stained with Alexa 568-labeled phalloidine (Molecular Probes, Eugene, OR), WASp with mAb 3D8.H5 (19), and Arp2/3 p34-Arc and p41-Arc with affinity-purified polyclonal Abs raised against whole recombinant p34-Arc and the C-terminal 10 residues of p41-Arc. (Note: all stainings and experiments described for p41-Arc have also been conducted for p34-Arc, yielding virtually identical results. Of p41-Arc staining (B), WASp staining (D), and overlays of A and B (C) and of D and E (F). Yellow color indicates colocalization. White bar indicates 10 μm.

FIGURE 1. Localization of actin, WASp, and p41-Arc in podosomes of primary human macrophages. Confocal laser scanning micrographs of ventral parts of cells, showing actin staining (A), WASp staining (D), p41-Arc staining (B and E), and overlays of A and B (C) and of D and E (F). Yellow color indicates colocalization. White bar indicates 10 μm.

was induced by exposing the cells to a gradient of fMLP (17) or by adding 1 μl of fMLP-solution (1 mg/ml, Sigma, St. Louis, MO) to the medium. The phenotypes of single polarized cells gained with both methods were indistinguishable. After a 6-h incubation, coverslips were removed and fixed in 3.7% formaldehyde.

Generation of WASp constructs and protein expression
WASp domain constructs were created by cloning PCR-generated inserts into the EcoRI and BamHI sites (in the case of construct A) or into the BamHI site (in the case of construct VC) of vector pGEX-2T (Pharmacia, Piscataway, NJ). Inserts of constructs were checked for correct orientation and fully sequenced. Proteins were expressed in Escherichia coli as GST fusions as described earlier (18). Purity was tested by SDS-PAGE and Coomassie staining.

Microinjection of proteins
Cells for microinjection experiments were cultured for 5–10 days. Microinjection was performed using transjector 5246 (Eppendorf) and a Compic Cells for microinjection experiments were cultured for 5–10 days. Microinjection was performed using transjector 5246 (Eppendorf) and a Compic

GST pull-down assay
A total of 6 × 10^6 cells cultured for 6 days in six-well plates (Nunc) at a density of 1 × 10^6 cells/well were washed with ice-cold PBS and lysed by addition of 200 μl/well of RIPA buffer (10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 140 mM NaCl) containing protease inhibitors. Cells were scraped on ice, and the resulting suspension was incubated for 30 min at 4°C with mixing. After centrifugation (15,000 rpm, 15 min, 4°C), aliquots of the supernatant were added to 400-μl aliquots of a 50% slurry of glutathione Sepharose beads, previously incubated for 1 h with 100 μg of GST-fusion proteins. Beads were incubated with lystate for 2 h at 4°C, washed two times with 1 ml of washing buffer (10 mM Tris/HCl, pH 7.5, 0.1% Triton X-100, 10% glycerol), and finally with 1 ml of washing buffer for 15 min at 4°C with mixing. Beads were pelleted, 100 μl of SDS-sample buffer was added, and an aliquot was run on a 12.5% SDS gel. Binding of proteins was tested by Western blot using the above-mentioned specific Abs. No binding of actin, Arp2/3 p34-Arc, or p41-Arc was detected when GST alone was bound to beads.

Results and Discussion
WASp has been shown to be an important regulator of podosome maintenance and organization (17). Additionally, WASp and WASp-like proteins can induce de novo actin nucleation and actin polymerization by binding to and activation of the Arp2/3 complex (20–22). Therefore, we reasoned that one if not the major mode of WASp-controlled podosome regulation is exerted via the Arp2/3 complex and that, in turn, a defective organization of the Arp2/3 complex may contribute to the phenotype of WAS macrophages. To test this hypothesis, we first performed immunofluorescence stainings of primary macrophages from healthy donors using Abs against p41-Arc and p34-Arc, two subunits of the Arp2/3 complex (23). Evidenced by costaining with actin and WASp, p41-Arc and p34-Arc specifically localized to the core of podosomes (Fig. 1). This suggests that the complete Arp2/3 complex is present in podosomes of human macrophages (see also Materials and Methods). Interestingly, another region of quiescent macrophages rich in actin filaments, the sub cortical actin ring, is seemingly void of the Arp2/3 complex (Fig. 1C). Instead, in quiescent macrophages the Arp2/3 complex is also present in the cytoplasm-rich but F-actin-poor region around the nucleus (see also Fig. 2, C and D).

Having shown that the Arp2/3 complex is a component of normal podosomes, we asked what the Arp2/3 complex localization in WAS macrophages may be. We were also interested in the question whether parts of this highly complex structures could be assembled independently of WASp and actin. When we stained macrophages from two molecularly well-characterized WAS patients (see Materials and Methods) using the anti-p41-Arc and -p34-Arc Abs, no podosomal staining of either of these two proteins could
be detected. Instead, both proteins were detected especially in the cytoplasm around the nucleus and also in the cell periphery (Fig. 2, A and B). These findings demonstrate that the Arp2/3 complex specifically colocalizes with actin and WASp in the core of macrophage podosomes and that intact WASp is necessary and sufficient to target the Arp2/3 complex to a podosomal localization where these proteins presumably assemble podosomal actin.

Additional evidence for this conclusion comes from microinjection experiments with proteins of podosome-disruptive ability. Microinjection of primary human macrophages with constitutively active V12CDC42Hs, which also leads to filopodia formation, or of a C-terminal WASp fragment encompassing the verprolin-like and central C-terminal domains (termed “VC domain”; Fig. 3A), resulted in the release of p41-Arc and p34-Arc from podosomes (Fig. 2, C and D). In most cases, the p41-Arc and p34-Arc staining became diffuse in the cytoplasm and was not associated with either filopodia or actin clumps. Dislocation of the podosomal components WASp, actin, and vinculin by microinjection of V12CDC42Hs or a C domain (17) is therefore also accompanied by dislocation of the Arp2/3 complex.

WASp and most WASp family proteins contain a short C-terminal stretch of acidic residues that is required for binding and activation of the actin polymerization activity of the Arp2/3 complex (termed “A domain”; Fig. 3A; Ref. 10). Additionally, a C-terminal fragment of the WASp homologue Scar1/WAVE1 (6), which includes the acidic domain, has been used to competitively block endogenous ActA-Arp2/3 interaction in mouse brain lysates (24). Based on these data and our findings that 1) the Arp2/3 complex is a component of podosomes and 2) WAS macrophages that lack intact WASp do not contain podosomes, we reasoned that removal of the Arp2/3 complex from podosomes and/or WASp should disrupt these structures. We thus injected macrophages with a GST fusion protein containing the 15 C-terminal amino acids of WASp (GST-A; Fig. 3A). In vitro pull-down assays showed that

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Podosomal localization of p41-Arc is absent in WAS macrophages and can be disrupted by microinjection of V12CDC42Hs or WASp VC domain in macrophages from healthy donors. Immunofluorescence micrographs of primary human macrophages, all cells stained against p41-Arc. A and B, Macrophages from WAS patients 1 (A) and 2 (B). C, Cell injected with V12CDC42Hs. D, Cell injected with WASp VC domain. Injected cells are indicated by star symbol. White bar indicates 10 μm.

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** A, Domain organization of WASp: WASp homology domain 1 (WH1), GTPase-binding domain (GBD), polyproline domain, C-terminal domain (containing verprolin-like and central C-terminal (“VC”) and acidic (“A”) domains). Numbers indicate first and last amino acid of WASp domains as part of the GST fusion proteins of VC and A domain. B, Actin and p41-Arc binding of VC domain and A domain fusion proteins. Western blots developed with anti-actin Ab (upper panel) or anti-p41-Arc Ab (lower panel). Molecular mass is indicated on the left. C and D, Control: microinjection of GST into primary human macrophages shows no effect on the localization of the Arp2/3 complex. C, p41-Arc staining. D, Staining against coinjected rat IgG. E and F, Microinjection of the Arp2/3-binding A domain of WASp into primary human macrophages leads to podosome disruption and actin ruffling. Immunofluorescence micrographs of primary human macrophages. E, p41-Arc staining. (Note: Arp2/3 is recruited to ruffles, but due to the difference in cytoplasmic content between the dome-shaped central and the flat surrounding parts of the cells and the resulting apparent accumulation of the complex around the nucleus, this may not be readily visible; Arp2/3-containing ruffles are especially visible in upper right part of injected cell.) F, Actin staining. Injected cells are indicated by star symbol. White bars indicate 10 μm for all panels of the same sizes.
GST-A was in fact able to bind to the Arp2/3 complex, whereas it did not bind actin (Fig. 3B). Injection of GST-A not only caused dislocation of the Arp2/3 complex (Fig. 3C), but also that of actin, WASp, and vinculin from their podosomal localization (Fig. 3F and not shown), consistent with a complete disruption of the podosomal structure. Interestingly, we also observed a strong ruffling response (Fig. 3F), which points to activation of actin polymerization in the cell periphery. Taken together, these findings show that the Arp2/3-binding domain of WASp can disrupt podosomes, but by a different mechanism than the WASp VC domain that needs its actin-binding ability (Fig. 3B) for this effect (17).

WAS monocytes and macrophages have been reported to show defects in the response to chemoattractants (15). In fact, it has been speculated that WAS may be a cell-trafficking disorder in which immune cells fail to translocate efficiently in response to inflammatory signals (16). The chemotaxis defect seems to stem from the inability of WAS immune cells to answer to chemotactic stimuli with correct polarization of the cell body (16), resulting in unoriented movement. We reported that polarization of primary macrophages involves elongation of the cell body and compartmentalization of podosomes and filopodia to the trailing and to the leading edge, respectively (17).

To elucidate the potential role of the actin-nucleating Arp2/3 complex in macrophage polarization, we microinjected the GST-A domain and then stimulated these cells with the chemoattractant fMLP. In parallel, uninjected control cells were fMLP-stimulated. Control cells and GST-A-injected cells were checked for typical signs of polarization: 1) presence of podosomes at the trailing edge, 2) elongated cell shape (ratio of length to breadth ≥ 2), and 3) presence of filopodia (Fig. 4A). We found that microinjection of GST-A not only reduced the number of cells containing podosomes (22% of GST-A-injected cells vs 94% of uninjected and 93% of GST-injected cells) but also the number of cells developing filopodia (18% of injected cells vs 58% of uninjected and 53% of GST-injected cells), whereas the number of elongated cells was not significantly altered (40% of injected cells vs 32% of uninjected and 30% of GST-injected cells; Fig. 4F). Therefore, macrophages injected with GST-A seem to be able to answer to fMLP stimulation with cell elongation to the same degree as control cells. However, their ability to form and reorient podosomes and filopodia was greatly reduced (Fig. 4C). The latter phenotype is reminiscent of fMLP-stimulated WAS macrophages that can still elongate but contain no podosomes and are at best able to form a few filopodia per cell (Fig. 4B). Additionally, the strong ruffling activity induced by injection of GST-A into primary macrophages is consistent with a well-preserved ruffling response observed in WAS macrophages (Fig. 4B) and WAS dendritic cells, the latter also being unable to develop a fully polarized cell morphology upon fMLP stimulation (13). The still existing potential of WAS macrophages for ruffling is not contradictory to a former study reporting a general smoothing of the surface of WAS lymphocytes (25). Generation of abnormally short and few microvilli on the apical surface of WAS lymphocytes can be compared with the loss...
of podosomal protrusions on the ventral surface of WAS macrophages, as both phenomena are results of a defective actin cytoarchitecture. Concurrent with this, in quiescent WAS macrophages also the apical surface seems to display fewer protrusions when compared with the apical surface of macrophages from healthy donors.

In sum, the WASp A domain seems to have a significant influence via its Arp2/3 binding ability on the chemotaxis response of macrophages. However, correct cell polarization is certainly not only dependent on Arp2/3 recruitment but also on external cues governing actin polymerization and thus needs the cooperation of other WASp domains like the GTPase binding domain, by which WASp is activated, or the proline domain, which mediates interaction with tyrosine kinases. Mutations in these domains may therefore also result in polarization impairment. Furthermore, mis-sense mutations that cluster in the amino-terminal WH1 domain generally lead to a milder WAS phenotype (2) but may still interfere with correct recruitment of WASp to the cell membrane and thus lead to similar, if potentially less severe, Arp2/3 recruitment defects.

Taken together, our data provide evidence that WAS macrophages are unable to target the Arp2/3 complex to a podosomal localization, which very likely contributes to their failure to assemble these adhesion structures. In addition, the effect of the isolated Arp2/3-binding GST-A domain on stimulated macrophages resembles the known phenotypes of stimulated WAS macrophages and WAS dendritic cells. These observations point to a critical role of the Arp2/3 complex in cell polarization of immune cells and a pivotal role of the Arp2/3 complex in the clinical manifestations of WAS.

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