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WASp and WAVE Proteins: From Structure, Through Function, to Clinical Aspects

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Abstract

T cells play a pivotal role in adoptive immunity, both in cell mediated cytotoxicity and in the activation of the humoral immune response. In order to perform their effector function, T cells undergo dramatic morphological changes upon activation. These changes enable their binding to and extravasation through the vascular endothelium into the neighboring tissue, the formation of an immunological synapse (IS) with an antigen-presenting cell (APC), and subsequently, the polarized secretion of cytokines and/or cytolytic granules, leading to the execution of effector functions.

The actin cytoskeleton is directly involved in all these processes. Thus, it is crucial for T cell mediated immune responses, providing a dynamic and flexible platform for signal transduction, cellular and subcellular remodeling, and for driving effector functions. The actin-regulatory proteins, Wiskott-Aldrich syndrome protein (WASp) and WASp family Verprolin-homologous protein (WAVE) play key roles in T cell biology. In this review, we will focus on these two proteins, describing their structure, recruitment, activation and function. Finally, we will address pathological aspects related to defects in these actin regulators.

Keywords: WASp; WAVE; Actin cytoskeleton; TCR; Signaling; Lymphocyte

Introduction

Immune response requires extensive trafficking of leukocytes in the blood stream, lymphatics and non-lymphatic tissues; this is enabled by dynamic cytoskeletal rearrangements. Recruitment of immune cells to inflammatory sites is dependent on dynamic cell shape changes to allow cell mobility from the blood stream, through the vascular endothelium into the underlying tissue. The rolling and tethering of immune cells in the blood vessels, and extravasation through the endothelium, require the actin cytoskeleton rearrangement. At the inflamed tissue, the phagocytosis of antigens by antigen-presenting cells (APCs) requires protrusion and retraction of the cell membrane, processes which are regulated by the actin cytoskeleton [1]. When naïve T cells engage APCs, dynamic cytoskeletal rearrangements support the formation of the immunological synapse (IS), during T cell priming. Armed T cells subsequently home to the inflamed tissue, where again the actin cytoskeleton supports the polarized secretion of cytokines and/or lytic granules and the execution of effector functions [2].

WASp and WAVE belong to the WASp family of actin nucleation promoting factors (NPFs), which includes (1) WASp, (2) neural-WASp (N-WASp), (3-5) WAVE proteins 1-3, (6) WASp and SCAR homolog (WASH), (7) junction mediating regulatory protein (JMY), and (8) WASp homolog associated with actin, membranes and microtubules (WHAMM). Within this family, WASp is exclusively expressed in hematopoietic cells, and WAVE2 is predominantly expressed in T cells [3].

Missense mutations, molecular deletions, or frame shifts in the *WAS* gene, which encodes WASp, result in Wiskott-Aldrich syndrome (WAS), an X-linked hematological disorder characterized by immunodeficiency, eczema, and thrombocytopenia, and associated with a high incidence of auto-immunity and lymphoid malignancies. Hereditary X-linked thrombocytopenia (XLT) is a milder variation caused by mutations reducing but not abolishing WASp expression [4,5].

Similar to other members of this family, WAVE2 and WASp possess a common verprolin homology, central hydrophobic region and acidic region (VCA) domain at their C-terminus. The VCA domain promotes actin polymerization through binding and activation of the actin-related proteins 2/3 (Arp2/3) complex [6], an actin nucleation complex that produces branched networks of filamentous actin (F-actin), commonly forming flat and thin membrane structures called lamellipodia and filopodia, respectively [7].

In addition to having similar VCA domains, both WASp and WAVE2 contain proline rich domains (PRD) and basic regions (BR), that enable their interactions with multiple Src-homology 3 (SH3)-containing proteins and with membrane-bound phosphoinositides, respectively. However, the two NPFs differ in their N-terminal domains, which facilitate their assembly into distinct macromolecular complexes. These complexes are pivotal for controlling the spatial and temporal cellular distribution of WASp/WAVE2, and for proper regulation of VCA- mediated actin polymerization activity. WASp contains an N-terminal WASp-homology 1 (WH1) domain, facilitating its interactions with WASp-interacting protein (WIP), and a GTPase-binding domain (GBD) that auto-inhibits the VCA by binding to it intramolecularly, while WAVE2 possesses an N-terminal WAVE-homology domain (WHD). Through its WHD, WAVE2 constitutively associates with four additional proteins: PIR121 (Sra-1), Hem-1 (Nap-1), Abi-1/2 and HSPC300, forming a pentameric complex also termed

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as WAVE regulatory complex (WRC) [8,9]. Furthermore, the presence of WASp and WAVE2 in macromolecular complexes have a vital role in their stabilization; in T lymphocytes the majority of WASp is found in complex with WIP, which protects WASp from degradation [10-12].

We have recently demonstrated the molecular mechanisms underlying WASp degradation by ubiquitylation [13]. We have shown that WASp undergoes ubiquitylation following T-cell antigen receptor (TCR) activation. This ubiquitylation is carried out by the E3 ubiquitin ligases Cbl-b and c-Cbl and is dependent on WASp activation and phosphorylation at tyrosine 291. Lysine residues 76 and 81, located at the WASp WH1 domain, which is the WIP binding region, were identified as the ubiquitylation sites, and disruption of these ubiquitylation sites results in WASp accumulation, altered actin dynamics, and the formation of actin-dependent structures. Thus, it seems that WIP masks and protects WASp ubiquitylation sites, and protects WASp from degradation either by calpain (cysteine protease) [10,14] or ubiquitylation [13]. Strikingly, most WASp gene missense mutations that disrupt the WIP-WASp interaction and usually result XLT [4], are located in the vicinity of WASp ubiquitylation sites. Unlike the WASp-WIP complex, the members of the WAVE complex are interdependent, protecting each other from degradation [15,16], though the mechanisms controlling WAVE stability are poorly understood.

Both WASp and WAVE2 localize to the IS following T cell antigen receptor (TCR) activation, where they facilitate actin cytoskeleton rearrangement, i.e. the formation of lamellipodia [9,17,18]. Additionally, WASp and WAVE families regulate rearrangement of F-actin, leading to cell migration, vesicular trafficking, endocytosis, and TCR-induced integrin activation [17,19-21]. WASp is also involved in the regulation of nuclear factor of activated T cells (NFAT) transcription factor, though in an unknown mechanism [22-25]. While the WASp deficiency was shown to cause WAS and the related XLT, WAVE2 deficiency is embryonic lethal, as mice deficient in WAVE2 die at approximately embryonic day 10 as a result of haemorrhages, caused by the lack of lamellipodia formation [26,27]. Thus, the different clinical manifestations of WASp and WAVE2 deficiencies or their overexpression imply distinct modes of regulation.

In this review, we will discuss the molecular mechanisms regulating WASp and WAVE2 and then examine WASp and WAVE2 effector functions and related diseases, and current therapeutic approaches.

Recruitment and Activation Mechanisms of WASp and WAVE2

The substantial differences between WAVE2 and WASp, two structurally related actin-regulating proteins, stem from their distinct regulatory mechanisms. Therefore, in order to understand how these two NPFs control different cellular functions and are involved in different diseases we must first analyze their molecular regulatory mechanisms.

Recombinant purified WASp is intrinsically inactive [28]. In resting T cells as well as other hematopoietic cells, WASp mainly exists in an autoinhibited state in which its C-terminal VCA domain intra-molecularly interacts with a hydrophobic core within its GBD, thereby interfering with its binding to the actin machinery [29]. Thus, WASp activation requires the disruption of its auto-inhibitory conformation and the release of the VCA domain from the GBD. Competitive binding of GTP-bound Cdc42 to WASp GBD results in conformational changes, releasing the VCA and enabling its interaction with the Arp2/3 complex, thereby promoting actin polymerization.

As WASp in T cells is mostly auto-inhibited, a sequential orchestrated chain of events takes place within the cell following TCR engagement, to enable its activation and recruitment (Figure 1) [30]. Among these events, the phosphorylation of the linker for activation of T cells (LAT) by the Syk-kinase zeta chain-associated protein of 70 kDa (ZAP70) provides a link between upstream signaling of TCR proximal kinases to downstream signaling events. The membrane-bound, phosphorylated LAT then binds, through the adaptor protein Gads, to the triple molecular complex of SLP-76, Nck and the guanine nucleotide exchange factor (GEF), VAV [31]. While Nck recruits WASp to the TCR site, VAV facilitates the activation of the Rho family GTPase, Cdc42. Then, activated Cdc42 mediates the activation of WASp [32]. Ultimately, WASp activation induces actin nucleation by Arp2/3, at the T cell-APC interface and the subsequent formation of the IS [33,34].

In addition to VCA release by Cdc42-GTP, in order to be fully activated, WASp requires the integration of additional signals. TCR ligation also results in the phosphorylation of WASp, resulting in its further activation. WASp phosphorylation by the Src family tyrosine kinases on tyrosine 291 has been well documented and demonstrated, both *in-vivo* and *in-vitro*, to enhance its capacity to stimulate actin polymerization and NFAT transcriptional activity [23,35,36]. Recently, a direct association was found between the WASp N-terminal domain and the SH3 domain of the Src family tyrosine kinase, Fyn *in-vitro* [37]. In the same study, blocking the interaction between this protein tyrosine kinase (PTK) and WASp impaired IL-2 production in activated T cells from transgenic mice. Furthermore, tyrosine phosphorylation of WASp and nuclear translocation of NFAT following TCR stimulation were severely inhibited by disrupting the WASp/Fyn interaction [37]. These data suggest that in T cells, WASp binding to Fyn through its N-terminal domain leads to WASp phosphorylation.

An additional factor that contributes to WASp activation is the membrane lipid, phosphatidylinositol (4,5)- bisphosphate (PIP2). This factor was shown in non-hematopoietic cells to bind the basic region of WASp and N-WASp, cooperating with Rho GTPases to facilitate both the activation and recruitment of WASp near the plasma membrane [38,39]. Therefore, it is of great interest to explore the validity of this mechanism in activated hematopoietic cells, e.g. T cells, for triggering WASp-dependent actin polymerization and cellular activation. The PRD of WASp is also important for the activation and recruitment of WASp to sites of actin polymerization; by binding SH3 domain-containing proteins such as Nck, the PRD facilitates enhanced interaction of WASp (as well as N-WASp) with its activators, via its recruitment to signaling complexes at the plasma membrane [17,40-42]. Accordingly, in a recent study of non-hematopoietic cells that combined quantitative analysis, fluorescence imaging, and computational modeling, it was demonstrated that as the local density of Nck SH3 domains increases, actin polymerization increases in a nonlinear manner. Moreover, computational predictions and experimental results suggested that the nonlinear dependence of actin polymerization on Nck density is the result of a previously unappreciated 4:2:1 Nck/N-WASp/Arp2/3 mechanistic stoichiometry. One Nck molecule was proposed to directly bind (via all three SH3 domains) the PRD of N-WASp, whereas the second Nck molecule indirectly contributes to the formation of an active

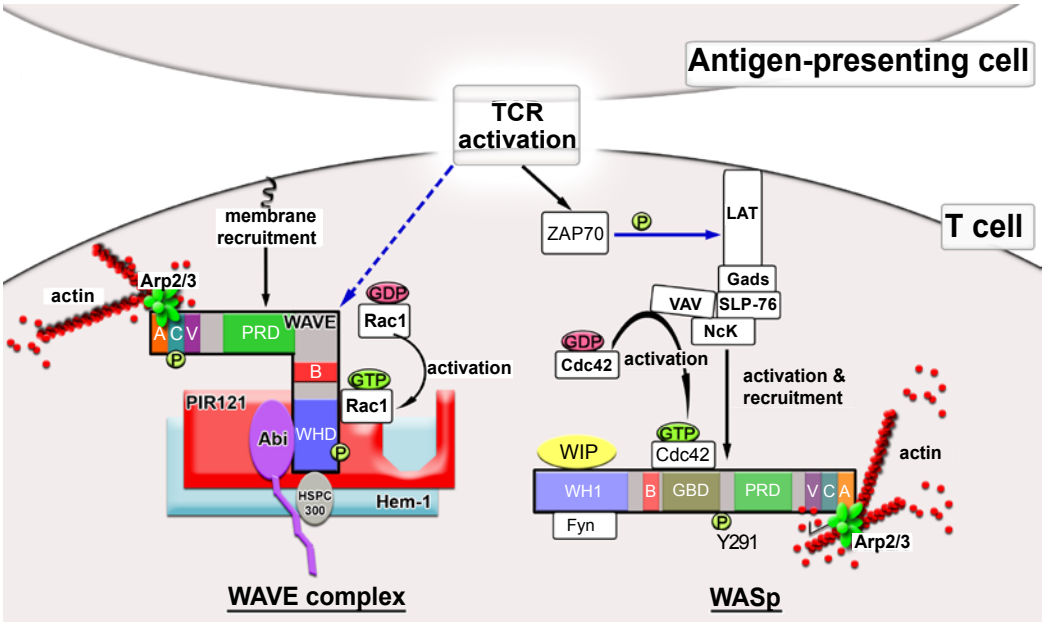

Figure 1: WASp and WAVE interactions following TCR activation. A sequential orchestrated chain of events takes place within the cell following TCR engagement: LAT adaptor protein phosphorylation by ZAP70, leads to the recruitment of the SLP-76, Nck and VAV triple molecular complex. Nck recruits WASp (*Right*) to the TCR site, while VAV activates the Rho GTPase Cdc42. GTP-bound Cdc42 then binds WASp, releasing WASp from autoinhibition. Additional phosphorylation at tyrosine 291 by the protein tyrosine kinase (PTK) stabilizes WASp active state. Subsequently, WASp mediates Arp2/3-dependent branched actin polymerization. The molecular mechanisms of WAVE activation are poorly understood. WAVE is regulated as part of a hetero-pentameric complex that includes: PIR121 (Sra1), Hem-1 (Nap1), Abi and HSPC300. WAVE mediates Arp2/3-dependent actin polymerization. Three requirements are necessary for the recruitment and activation of the WAVE complex (*Left*): (1) Binding the Rho GTPase, Rac1, which can be activated by VAV (2) binding to lipid vesicles containing acidic phospholipids, i.e. PIP3 and (3) a specific mode of phosphorylation.

N-WASp complex through an interaction (via the second Nck SH3 domain) with WIP (which is constitutively bound to N-WASp) [43]. This model and the proposed stoichiometry are also consistent with the previously reported requirement for dimerization/oligomerization for the enhancement of N-WASp *in-vitro* actin polymerization activity by SH3 domain–containing proteins [38].

While the molecular mechanisms underlying WASp activation and recruitment to the TCR site were extensively studied, those regulating WAVE2 remain mostly unknown.

Unlike WASp, members of the WAVE sub-family lack a GBD and do not exist in an auto-inhibited conformation. Instead, recombinant purified WAVE1 and WAVE2 proteins are constitutively active [44,45] and are trans-inhibited by their constitutive interactions with the pentameric WRC [44,46,47]. Being a hetero-pentameric complex, the WRC has been difficult to characterize, and its basal activity state has been a subject of debate [48,49]. Initially, the WRC was found to be basally inactive, and its binding to Rac1 and Nck was suggested to trigger its dissociation and subsequent WAVE1–dependent actin nucleation [47]. However, other groups showed that the WRC is basally phosphorylated and active [16,50], thereby attributing its spatial and temporal actin nucleation activity mostly to its localization. Later studies resolved most of the controversy and determined, using advanced purification and reconstitution techniques, those as part of the WRC, WAVE1 and 2 are inactive and inhibited [44-46].

Among the putative WAVE activators, the small GTPase, Rac1, is the most fully characterized. Like Cdc42, Rac1 can be activated by VAV, and through WAVE, Rac1-GTP mediates the formation of membrane structures [15,26,51-53]. In a comprehensive study of *in-vitro* actin polymerization activity of the WAVE2 complex, three requirements were found necessary for activation of the native complex: (1) Binding to prenylated Rho GTPase Rac1-GTP, (2) binding to lipid vesicles containing acidic phospholipids, and (3) a specific mode of serine/threonine phosphorylation. Moreover, these inputs must be applied concomitantly for the complex to be activated (Figure 1) [45].

Nevertheless, although the key factors that contribute to the recruitment and activation of the WRC were identified, the molecular mechanism of this activation remains elusive. Analysis of the WRC crystal structure [8] hinted at a possible molecular activation mechanism of WAVE proteins. Based on this structural analysis, the VCA domain of WAVE1 is sequestered by residues 82-184, to a conserved niche within Sra1, rendering it inaccessible to the Arp2/3 complex. The binding of Rac1 to Sra1, in a proximate position to the interface with the VCA, competes with the binding of the VCA to WAVE itself and to Sra1, thereby inducing a conformational change that facilitates the release of the VCA [8]. In agreement with these *in-vitro* studies, phosphorylations of tyrosine 151 (equivalent to Tyr150 of WAVE2) and tyrosine 125, located at the interface between WAVE1 and Sra1, and phosphorylations of tyrosines 150 and 124 of WAVE2 were shown to upregulate lamellipodia structure formation, suggesting activation of the WRC by these two tyrosines [8]. Moreover, charge distribution mapping of the entire WRC surface revealed a polarized arrangement of this complex, which is proposed to orient the complex to bind the membrane through the interactions between the acidic phospholipids e.g. phosphatidylinositol (3,4,5)-triphosphate (PIP3), and the positively charged face of the complex; such an orientation exposes the negatively charged face (including the VCA domain) to the cytoplasm, where actin polymerization takes place [8]. It must be noted

that in order to facilitate crystallization of the WRC, the C-terminal proline-rich region and SH3 domain of Abi2 were genetically deleted, and the PRD of WAVE1 was replaced with an 18-residue linker. Therefore, the possible role of SH3 containing proteins in WAVE activation could not be evaluated in this system, and therefore remains a subject for further investigation. Finally, the proposed mechanism of WAVE activation, based on this structural data, is yet to be verified *in-vivo*, and is likely to be a subject of intensive research in the near future.

Similar to WASp, WAVE2 has also been suggested to be regulated by phosphorylation. In non-hematopoietic cells, the tyrosine kinase, c-Abl, has been shown to bind the WAVE2 complex member Abi-1, and to directly phosphorylate WAVE2 at tyrosine 150, leading to enhanced WAVE2-dependent actin polymerization [54,55]. In T cells, however, no phosphorylation of WAVE2 tyrosine 150 could be detected [9,56]. Although a c-Abl mediated direct phosphorylation of WAVE2 in T-cells has not been demonstrated, c-Abl was found to be essential for appropriate WAVE2 recruitment to the IS [56]. It is possible that c-Abl contributes to WAVE2 activation and recruitment indirectly, by phosphorylation of other members of the WAVE2 complex, such as Abi, a known substrate of c-Abl [37,56,57]. In non-hematopoietic cells, five serine residues, located at the VCA domain, were shown to be phosphorylated by Casein Kinase 2 (CK2) *in-vitro*. Phosphorylation of these sites is required for a high affinity interaction with the Arp2/3 complex, and two of these phospho-serines inhibit the activation of the Arp2/3 complex by the WAVE2 VCA domain [58]. Supporting these data, WAVE2 serine/threonine phosphorylation has been detected, following TCR engagement, downstream of Erk and PKCs [9]. These data suggest that the phosphorylation of WAVE2 could be a possible mechanism to control WAVE2 activation in T cells, though further research is required to validate the significance of this process. The Phosphatidylinositol 3-kinase (PI3K) product, PIP3 has also been suggested to contribute to WAVE membrane recruitment and activation. PIP3 was shown to directly bind the basic region of WAVE, to recruit the WAVE complex to the plasma membrane, and to cooperate with Rac1 in its activation.

As TCR ligation results in both the activation of Rho GTPase and Src kinases, along with the clustering of membrane phosphoinositides, it is possible that the fine tuning of WASp and WAVE2 activation is achieved by a combination of these mechanisms.

WASp and WAVE2 Effector Functions in T cells

WASp and WAVE2 share similar functions

Both WASp and WAVE2 are recruited to the IS following TCR activation, where they facilitate the formation of actin-rich structures, which are essential for T cell migration and scanning of target cells/APCs. Both proteins are involved in IS formation or/and stabilization [59]. They were shown to be required for T cell activation, as absence of either of these proteins impairs IL-2 production, NFAT transcriptional activity and calcium flux [9,22,25,60]. Furthermore, WASp-deficient mice exhibit impaired T cell differentiation [22,61], and although there is no WAVE2-deficient mouse model (due to embryonic lethality), there are some evidences from other mouse models which support such involvement [62], as will be described later.

Although WASp and WAVE2 share similar molecular structure and some cellular activities, they also control distinct cellular functions (Figure 2).

WASp links actin rearrangement with endocytosis

Early studies of T cells from WASp-deficient mice have shown substantial impairment of TCR endocytosis, following ligand binding [22]. In agreement with these studies, WASp was found to be involved in the process of clathrin-dependent TCR endocytosis [19,22]. This process includes the assembly of clathrin-coated membrane pits via the adaptor protein complex, adaptor protein-2 (AP2). AP2 interacts directly with motifs in the cytoplasmic tails of activated transmembrane receptors, and with membrane-bound PIP2, as well as other auxiliary proteins. Endocytic vesicle scission is then mediated by the GTPase, dynamin, whose polymerization around the neck of the nascent vesicle triggers GTP hydrolysis and consequent membrane fission [63]. In T cells, the endocytic adaptor protein, intersectin-2, binds both WASp and Cdc42, following TCR activation, facilitating their activation and recruitment, thereby linking WASp activation and actin rearrangement with its translocation to endocytic vesicles [19]. Intersectin-2 interacts

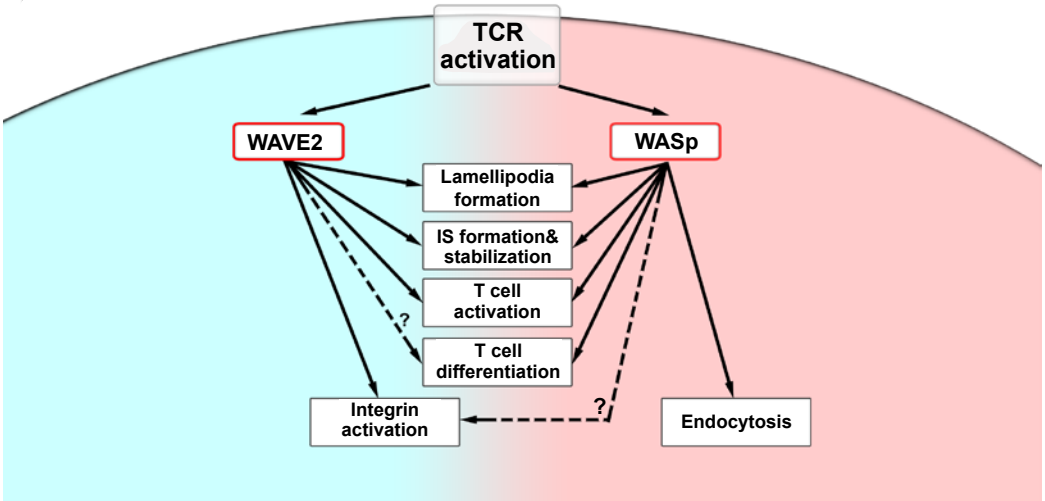

Figure 2: Schematic representation of WASp and WAVE2 common and distinct function in T cells. WASp (Right) and WAVE2 (Left) control both common (Middle) and distinct cellular processes. Arrows with broken lines indicate speculative involvement.

with numerous components of the endocytic pathway, including clathrin, AP2 and dynamin [64]. Interestingly, similar to the WASp-Nck interaction, WASp associates with intersectin-2 through binding of its PRD to multiple SH3 domains within intersectin-2 [19].

In addition to WASp-mediated TCR endocytosis, WASp also mediates internalization of the CD28 co-receptor in an activation-dependent manner, through an interaction with sorting nexin 9 (SNX9), an endocytic protein also involved in membrane trafficking and protein sorting [65]. SNX9 has high affinity for AP2 and clathrin, and thereby enables the efficient recruitment of dynamin to endocytic vesicles [66]. SNX9 synergizes with PtdIns (4,5) P2-containing liposomes to augment the GTPase activity of dynamin [67]. Following TCR/CD28 costimulation, SNX9 forms a multimeric signaling complex that includes WASp, the phosphatidylinositol (PtdIns) 3-kinase (PI3K) p85 regulatory subunit, and CD28, which are colocalized within endocytic vesicles. This endocytic complex formation is mediated by binding of the SNX9 SH3 domain with WASp, and the SNX9 phox (phagocyte oxidase) homology domain (PX) with both p85, and the PIP3 product of PI3K. Thus, SNX9 is suggested to enable WASp coupling to PI3K, PIP3, and CD28 and thereby promote ligation-induced CD28 internalization and downstream PI3K-dependent signaling events [65].

Imaging studies in live activated T cells demonstrated the co-internalization of WASp with the adapter protein, SLP-76 [17], a central coordinator of actin polymerization. These data demonstrate that WASp is a central regulator of T cell receptors and cytoskeletal proteins endocytosis. The role of WASp as a linker between T cell activation to actin polymerization and endocytosis makes WASp an important player in the process of T cell signaling attenuation.

WAVE2 regulates integrin-mediated adhesion

In T cells, WAVE2 was found to be a major regulator of integrin activation, facilitating clustering and activation of integrins, in a TCR activation-dependent manner. Accordingly, increased integrin function is associated with over expression of the WRC member PIR121 in $CD4^+$ T cells isolated from multiple sclerosis patients. Thus, these data demonstrate that the WAVE2 complex has an important role in the localization and activation of central integrin activating proteins [68].

Integrins are a large family of αβ heterodimeric adhesive cell surface proteins that promote cell to cell interactions and interactions of cells with extracellular matrix proteins [69]. In resting T cells, integrins exhibit low affinity and avidity for their cognate ligands. In order to enhance their ligand-binding, integrins undergo conformational changes and clustering that enable their efficient activation [70], and as a consequence, the formation of a stable IS [34]. The process of integrin conformational change and clustering is dependent in part on a series of TCR-triggered signaling events termed "inside out signaling" [71].

WAVE2 integrates into the integrin inside-out signaling pathways at several points. Following T cell activation, WAVE2, through its VCA domain, forms a complex with Arp2/3 and the integrin binding proteins, vinculin and talin, thereby recruiting talin to the IS, and linking actin polymerization with integrin activation [20]. Another point of integration is through the small GTPase Ras-proximity-1 (Rap1), a potent activator of integrins in lymphocytes [72]. The WAVE2 complex was shown to activate Rap1 through the recruitment of the Abl tyrosine kinase and a complex of the CrkL adaptor protein with C3G (GEF of Rap1) to the IS, independently of its actin polymerization activity. At the IS, Abl phosphorylates and activates C3G, which in turn activates Rap1 [21]. These data suggest that WAVE2-dependent integrin clustering (through vinculin and talin) and integrin activation (through Rap1 activation) are separate processes, in which the clustering depends on actin polymerization while activation, does not. Therefore, it would be interesting to follow the dynamics of these two processes and the players involved, in live activated T cells. Moreover, it was shown that Rap1 could still be partially activated in the absence of WAVE2, and that Rap1 activation is blocked by the actin depolymerizing agents, cytochalasin D or latrunculin B [21]. Thus, an alternative, actin-dependent and WAVE2-independent pathway may lead to Rap1 activation.

Interestingly, WASp was shown to control integrin activity in natural killer (NK) cells. Chemokine activated NK cells from WAS and XLT patients exhibit impaired integrin adhesion, and endothelial cell-mediated migration. The defective chemokine-induced migration of these cells is in correlation with reduced expression of the activated form of β2 integrin, and decreased adhesion. Inhibition of WASp-Cdc42 binding, using wiskostatin (WASp activation inhibitor), had a similar effect on NK cells, inhibiting their chemokine-induced integrin-dependent migration and β2 activation. Thus, these data emphasize that the WASp/Cdc42 pathway is essential for NK cell integrin-mediated migration in response to chemokine receptor induced inside-out signaling [73]. To date, this is one of a few evidences of WASp involvement in integrin inside-out activation. Another study showed that WASp-deficient murine neutrophils and human neutrophils from WAS patients displayed substantial defects in integrin clustering and activation, these defects resulted from integrin evoked signaling (outside-in), rather than receptor induced (inside-out signaling) effects [74]. Nevertheless, although WASp might be involved in integrin activation through its actin polymerization activity, the molecular mechanisms regulating WASp-dependent integrin activation are unknown.

Disease Related Aspects of WASp and WAVE2

Mutations and deletions of the *WAS* gene can lead to a spectrum of clinical diseases (Figure 3):

(1) the classical WAS, a severe immunodeficiency characterized by thrombocytopenia, bleeding, eczema, recurrent infections, and susceptibility to the development of autoimmune diseases and lymphomas [75,76]

(2) the milder variant of WAS, XLT, which is caused by missense mutations reducing WASp expression, and characterized mainly by thrombocytopenia, and in some cases associated with mild eczema and infections [4]

(3) X-linked neutropenia (XLN) caused by activating mutations in the VCA binding region of the GBD, preventing normal intramolecular interaction with the C terminus, and leading to aberrant auto-inhibition of WASp and dysregulation of actin polymerization [77]. Since WASp is expressed selectively in hematopoietic cells and is involved in cell signaling and cytoskeleton reorganization, it has global effects on the functions of T and B lymphocytes, NK cells, dendritic cells (DC), and platelets [76,78]. Recently, a WIP deficiency was found in a patient with features of WAS that included eczema, recurrent infections, thrombocytopenia, T cell lymphopenia, impaired T cell proliferation to immobilized anti-CD3, defective T cell chemotaxis, and decreased NK cell function. Although this patient had no detectable levels of WASp, her *WAS* sequence and mRNA levels were normal, indicating that WIP deficiency was responsible for this immune dysfunction

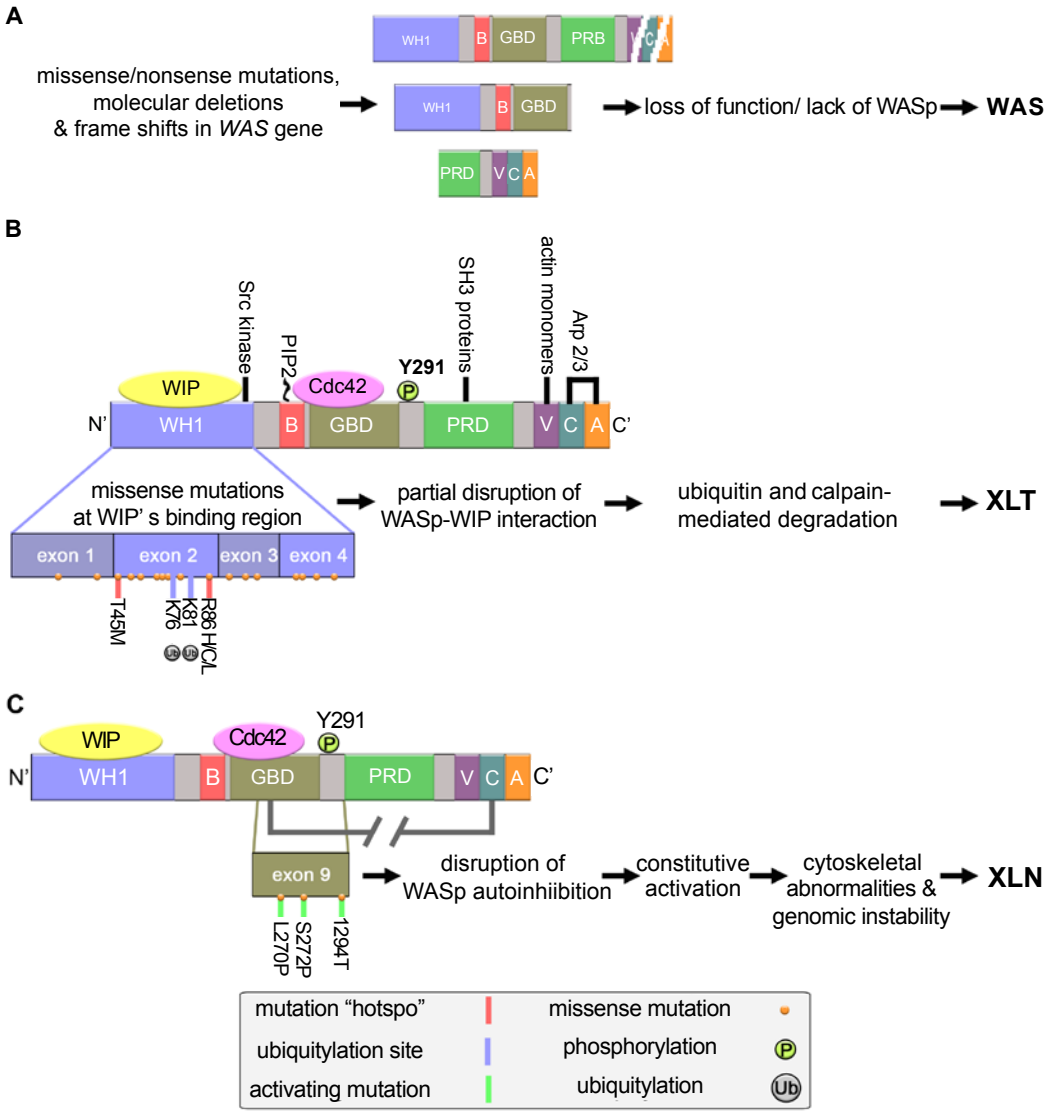

Figure 3: Molecular basis of WASp-related disease. Mutations and deletions of the *WAS* gene can lead to a wide spectrum of diseases: (**A**) WAS is resulted by various missense/nonsense mutations, deletions and frame shifts in the *WAS* gene, leading to WASp deficiency or dysfunction. (**B**) WIP protects WASp from ubiquitin and calpain protease-mediated degradation. Missense mutations at the WH1 domain of WASp disrupt the WASp-WIP interaction; WASp activation by the Rho GTPase Cdc42-GTP, and phosphorylation by protein tyrosine kinase, eventually result in WASp ubiquitylation at the lysine residues 76 and 81 and proteolysis by calpain. Enhanced degradation of WASp usually causes XLT. (**C**) XLN is caused by activating mutations in the VCA-binding region of the GBD, which disrupt WASp autoinhibition. Constitutive WASp activation leads to cytoskeletal abnormalities and genomic instability.

[79]. Interestingly, introduction of WIP to the patient's T cells rescued WASp expression by these cells, suggesting that the absence of WIP in the patient's T cells resulted in WASp instability and degradation. These results support previous evidence that WIP protects WASp from degradation [10,13,14]. Since the phenotypes of WIP and WASp deficiencies are similar, it was suggested that WAS cannot be diagnosed only by the level of WASp protein expression, but a DNA diagnosis must also be performed by sequence analysis of *WAS* and *WIP*.

The WAS primary immunodeficiency highlights the importance of WASp for T cell function. Analysis of WASp-deficient mice revealed T cell migration defects, as well as substantial decrease of peripheral blood T cell numbers, aberrant antigen receptor–induced proliferation and IL-2 production, and marked reduction in actin polymerization [22,60]. Additionally, while some studies reported T cell differentiation in WASp deficient mice to be impaired, as manifested by reduced transit from the double-negative (DN) to the double-positive stage (DP) and specifically from $CD44^{-}CD25^{+}$ (DN3) to $CD44^{-}CD25^{-}$ (DN4) expression [22], others reported T cell development to be normal [60]. These data could be explained by the compensating effect of other cytoskeletal proteins; accordingly, T cells from WASp and N-WASp double knock-out mice were impaired in their DN to DP transition [61]. Thus, it seems that WASp and its homolog N-WASp are both required for normal T cell development. Interestingly, T-cells of VAV-deficient mice have similar defects to those of WASp-deficient mice, i.e. impaired actin-cytoskeleton rearrangement, TCR clustering and IL-2 transcription, and developmental blockage [80-83]. The phenotypic resemblances between WASp and VAV deficiencies strongly suggest

that these proteins act at the same pathways, though WASp function has not been examined in the context of VAV-deficient animal model. Early studies with T cells of WAS patients have shown that TCR-induced proliferation and actin polymerization are both impaired [84-86]. In fact, defective IL-2 expression and T-cell proliferation in WAS, were found to be caused by lack of WASp dependent NFAT activation, nuclear translocation of p-Erk, Elk1 phosphorylation, and c-fos gene expression [25]. Thus, WASp was suggested to be essential for T cell activation and effector functions either by mediating transcriptional activity or by the polymerization of actin. Indeed, WAS patients exhibit high incidence of malignancies [76], further suggesting a malfunction in cellular-mediated immunity, as well as impaired immune surveillance.

The ability of T cells to produce and secrete Th1 and Th2 cytokines has been studied in the context of WASp deficiency. $CD4^+$ and $CD8^+$ T cells from WASp-deficient mice have impaired production and secretion of Th1, but not Th2 cytokines, under TCR/CD28 stimulating conditions [87,88]. Recently, WASp localization to the nucleus of human differentiating primary T helper 1 (T_H1) cells was demonstrated. Nuclear WASp was shown to engage Th1-specific genes, but not Th2 or T regulatory (Treg) genes, *in vivo* under T_H1-differentiating conditions. WASp epigenetically controls the transcriptional activity of the Th1 master regulator, T-BET, through its local effects on chromatin configuration and conformation [89]. Indeed, previous studies demonstrated a selective defect in the expression of T-BET in $CD4^+$ T cells isolated from WAS patients [87]. Furthermore, T_H cells, demonstrate attenuation of *TBX21* (T-BET gene) promoter dynamics, resulting an impairment of Th1 activation and function. Th1 functional defects could be rescued by reintroducing WASp [89]. Thus, in T cells, WASp involvement in transcriptional activity appears to be essential primarily for Th1-directed immune responses.

Another clinical aspect of WASp deficiency is autoimmunity. Autoimmunity is very common among WAS patients, as 40–70% of the patients are affected by at least one autoimmune disorder [90]. Being recognized as major negative regulators of autoimmune responses, regulatory T cells (Tregs) have been extensively studied in WASp-deficient mice and humans. Accordingly, WASp-deficient Tregs were demonstrated to have impaired homing to inflamed tissues, aberrant homeostasis and impaired suppressor function [90-93]. Several factors were suggested to contribute to this Treg dysfunction. First, WASp-deficient Treg suppressor function could be partially rescued by IL-2, whose production and secretion is WASp dependent [90,91,93]. Furthermore, IL-10 and TGF-β secretion were found to be impaired in WAS Tregs, suggesting an intrinsic role for WASp in Treg activity [91,93]. A recent study showed that $CD4^+$ T cells from WASp-deficient mice undergo reduced apoptosis following restimulation of the TCR. This correlated with reduced active Fas ligand (FasL), and granule secretion by WASp-deficient T cells [94]. Thus, WASp is suggested to have a major role in limiting autoimmunity, either by mediating Treg effector function or by being involved in the secretory pathway of FasL in re-stimulation-induced cell death (RICD) of self-reactive effector T cells.

The WAVE proteins, although sharing some structural and functional characteristics with WASp and its homolog N-WASp, greatly differ in their cellular function and related diseases. Unlike WASp, WAVE2 deficiency is embryonic lethal, due to haemorrhages, caused by aberrant actin rearrangement [26,27]. This indicates the crucial role of WAVE2 in actin machinery and fundamental cellular functions. Although WAVE2 deficiency is embryonic lethal, and thus WAVE2 general effects on the immune system could not be directly addressed, studies of mice deficient or mutated in other members of the WRC hinted on WAVE2 essential role in the immune system. Abi-1 deficiency is also embryonic lethal, probably due to the instability of the other members of the WRC [95]. Therefore, the successive establishment of a Hem-1 deficient mouse model, using a chemical mutagenesis strategy [62], provided valuable hints on the possible physiological consequences of WAVE2 deficiency. Hem-1 deficient mice exhibited lymphopenia, neutrophilia, and anemia. Specifically, T-cell development was impaired; T cells showed decreased activation and proliferation, displayed aberrant actin polymerization, and decreased integrin-mediated adhesion to fibronectin [62]. In agreement with other studies that showed the WRC proteins to be interdependent [16,95], loss of Hem1 resulted in the near complete degradation of the WRC members WAVE2, Abi1-2, and Sra1 both in thymocytes and T cells [62]. Thus, these defects could be because of the absence of WAVE2 (or other members of the WRC), though the involvement of WAVE2-independent pathways cannot be excluded. Nevertheless, it is of interest to establish a non-lethal WAVE2 deficiency mouse model in order to directly examine WAVE2 function in a physiologically relevant context.

Interestingly, some pathologies were suggested to involve other components of the WAVE pentameric complex. Recently, increased integrin function was shown to be associated with over expression of PIR121, a member of the WRC, in CD4 T cells isolated from multiple sclerosis (MS) patients. MS is the most common neurological disease of young adults associated with a chronic inflammatory response, demyelination and axonal loss. $CD4^+$ and $CD8^+$ T lymphocytes are present in high numbers in MS lesions [96]. It was observed that PIR121, which is a part of the WAVE1 complex, is upregulated in $CD4^+$ cells of MS patients, and this over-expression is correlated with increased integrin activation [68]. Reduction of PIR121 protein levels or inhibition of the WAVE activator, Rac1, caused a reduction in fibronectin-mediated binding of $CD4^+$ cells, suggesting that the MS phenotype is a result of WAVE-dependent activity, through a still-unknown mechanism. These results further suggest that controlling PIR121 gene expression or function may assist MS patients.

Recently, WAVE2 was found to be involved in HIV-1 viral infection. HIV-1 enters the cells by fusion at the plasma membrane or by endosomes, and it's binding to target cells, mediated by the envelope glycoprotein (Env), induces activation of Rac and stimulates actin cytoskeleton rearrangement [97]; thus, viral fusion can be inhibited by dominant negative Rac [98]. Arp2/3 is a downstream effector of Rac, which is activated by the WAVE2 complex. Accordingly, a recent paper demonstrated that the WAVE2 complex and the PTK, Abl, which phosphorylates WAVE2, are required for Env-mediated membrane fusion entry, and infection, and that Abl kinase inhibitors may have a role in the treatment of HIV-1-infected patients [99]. Current therapies are directed against the viral protein, which undergoes mutations that result in resistance to therapy. Therefore, targeting signaling proteins instead of viral proteins, may offer new strategies for treatment of infected patients who develop resistance to anti-viral therapies.

Current Therapeutic Approaches for WASp-related Diseases

Classical WAS results in premature death unless treated by splenectomy, bone marrow transplantation, cord blood transplantation or gene therapy. The treatment of choice depends on the severity of the immunodeficiency. Stem cell transplantation is the preferred

treatment, especially for young patients, whereas therapeutic options for XLT patients are a matter of debate [4]. It is important to select safe and effective individualized therapies for specific patients, taking into account other aspects such as age of the patient and the specific mutation in WASp [100].

Stem cell transplantation

Stem cell transplantation is successful when a related human leukocyte antigen (HLA)-identical donor is available, and leads to restoration of immune and haemostatic functions [76]. Transplants from semi-allogeneic related donors results in a lower percentage of survival [101], and in many cases, leads to high rates of graft rejection and lymphoproliferative diseases. There were also reports of successful transplantation using closely HLA-matched unrelated donors [102]. A recent case study demonstrated the use of busulfan, which resulted in good engraftment without serious complications, emphasizing the need to optimize transplantation protocols [103]. Hematopoietic stem cell transplantation (HSCT) can be successful, but may result in secondary malignancy and infertility. Lack of suitable donors and mismatched transplantation leads to high morbidity and mortality, and are associated with Epstein-Barr virus (EBV) lymphoproliferative syndrome, infections, autoimmunity, and graft versus host disease (GVHD). HSCT can be improved by earlier diagnosis, infection surveillance, and promotion of immune reconstitution [104].

Cord blood transplantation

Umbilical cord blood (CB) transplantation also offers a therapeutic option with a better outcome in younger patients [101]. CB appears to be an alternative donor source compared with matched unrelated bone marrow, with successful engraftment associated with absent to mild acute GVHD and with no development of chronic GVHD. A recent study demonstrated genetic correction as well as phenotypic correction by cord blood stem cell transplantation (CBSCT) in two male WAS patients [105].

Splenectomy

Splenectomy is one of the alternatives for treatment of XLT and WAS patients who are not candidates to transplantation. Splenectomy generally increases the platelet count but is not widely used, mainly because of post splenectomy complications [100] that may result in severe infections and sepsis and require life-long antibiotic treatment [4]. Nevertheless, splenectomy can mitigate the disease if a rigid follow-up program, and patient education is maintained [106].

Gene therapy

Previous studies show that gene therapy can offer an alternative treatment for patients who lack an appropriate stem cell donor. Patients with mutations predictive of severe disease may also benefit from gene therapy, since the milder phenotype of XLT demonstrates that even low level correction of WASp expression may be therapeutically effective [5]. *In vitro* studies on human WASp deficient B and T cell lines transfected with appropriate vectors demonstrated restoration of proliferative immune responses [107].

$WAS^{-/-}$ bone marrow cells were transduced with the WASp gene, and injected into $WAS^{-/-}$ recipient mice. After engraftment, leukocytes expressed WASp at similar levels as those of the WT control. Splenic T cells exhibited correction of the WASp proliferative defect and improvement in cytokine production defects [108]. Mice reconstituted with lentiviral vectors encoding an eGFP-WASp fusion protein exhibited long term restoration of migratory activity in myeloid and lymphoid lineages [109].

Nevertheless, since gene therapy involves transfer of the relevant transgene into autologous $CD34^{+}$ haematopoietic stem cells using a retroviral vector, the complications of the technology may result in leukemia or myelodysplastic changes. Novel approaches to improve the safety profile of viral vectors, and to optimize vector design are studied [110]. Novel assays are being developed in order to model the risk of insertional mutagenesis [111]. HIV-1-derived (rHIV) lentiviral vector was recently developed for WAS, presenting several advantages, and leading to the initiation of gene therapy trials, designed to demonstrate safe restoration of multilineage immunological function [5]. Such treatment requires a multicenter approach in order to facilitate an accurate protocol of treatment and a long term follow-up to examine clinical recovery.

Summary

Understanding the precise mechanisms by which WASp and WAVE are regulated is of great clinical importance, as activation or inactivation results in distinct diseases. The WAS/XLT/XLN immunodeficiencies have provided insights to the molecular mechanisms regulating WASp activation, recruitment and function. The genotype-phenotype correlations and the molecular basis for these diseases are now starting to be revealed. This constantly-expanding knowledge provides opportunities to shift from traditional treatments for WAS/XLT, i.e. splenectomy and HSCT, to gene therapy and custom-designed medications [112]. Moreover, understanding the mechanisms whereby WASp deficiency results in disease may enable more reliable diagnosis, and might help optimize treatment for each specific mutation. In contrast to WASp, the absence of a WAVE deficiency syndrome, due to its embryonic lethality, further emphasizes its important role in embryonic development on one hand, but on the other hand, provides fewer opportunities to reveal its molecular regulatory mechanisms. Nevertheless, the major role of WAVE2 in T cell function, as well as its involvement in HIV-1 viral infection, and its possible involvement in MS, makes this protein subject of intensive research. Following the recent structural characterization of the WRC, the foundation is laid for the discovery of its regulatory mechanisms. In the future, pharmacological intervention in the signaling cascades of WASp and WAVE may offer novel therapeutic strategies to treat cytoskeletal protein-related diseases.

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