RECENT ADVANCES IN UNDERSTANDING THE PATHOPHYSIOLOGY
OF WISKOTT-ALDRICH SYNDROME

Running title: WISKOTT-ALDRICH SYNDROME

Marita Bosticardo¹,², Francesco Marangoni¹, Alessandro Aiuti²,³, Anna Villa¹,⁴, and Maria Grazia
Roncarolo¹,²

¹San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), 20132 Milan, Italy; ²Università
Vita-Salute San Raffaele, 20132 Milan, Italy; ³University of Rome “Tor Vergata”, 00133 Rome,
Italy; ⁴CNR-ITB, 20090 Segrate, Milan, Italy

Correspondence and reprint requests should be addressed to: Maria Grazia Roncarolo, San Raffaele
Telethon Institute for Gene Therapy (HSR-TIGET), via Olgettina 58, 20132 Milan, Italy

Phone: (+39) 02.26434875; Fax: (+39) 02.26434668; E-mail: m.roncarolo@hsr.it
Wiskott-Aldrich syndrome (WAS) is a severe X-linked immunodeficiency caused by mutations in the gene encoding for WASP, a key regulator of signaling and cytoskeletal reorganization in hematopoietic cells. Mutations in WASP result in a wide spectrum of clinical manifestations ranging from the relatively mild X-linked thrombocytopenia (XLT) to the classic full-blown WAS phenotype characterized by thrombocytopenia, immunodeficiency, eczema, and high susceptibility to developing tumors and autoimmune manifestations. The life expectancy of patients affected by severe WAS is reduced, unless they are successfully cured by bone marrow transplantation from related identical or matched unrelated donors. Since many patients lack a compatible bone marrow donor, the administration of WAS gene corrected autologous hematopoietic stem cells could represent an alternative therapeutic approach. In the present review, we focus on recent progress in understanding the molecular and cellular mechanisms contributing to the pathophysiology of WAS. Although molecular and cellular studies have extensively analyzed the mechanisms leading to altered defects in T, B and dendritic cells, the basis of autoimmunity and thrombocytopenia still remain poorly understood. A full understanding of these mechanisms is still needed to further implement new therapeutic strategies for this peculiar immunodeficiency.
Introduction

Wiskott-Aldrich Syndrome (WAS, OMIM 301000) is a complex and severe X-linked disorder characterized by micro-thrombocytopenia, eczema, immunodeficiency and increased risk in developing autoimmunity and lymphomas. WAS affects 1 to 10 out of a million male newborns, whose life expectancy is about 15 years for patients lacking WASP expression\(^1,2\). The protein encoded by the \textit{WAS} gene (Wiskott-Aldrich Syndrome Protein, or WASP) is a hematopoietic specific regulator of actin nucleation in response to signals arising at the cell membrane\(^3,4\). Mutations impairing but not abolishing WASP expression, can cause X-linked thrombocytopenia (XLT). This disease can be chronic\(^5\) or intermittent\(^6\), and is considered an attenuated form of WAS since it is characterized by low platelet counts with minimal or no immunodeficiency. Recently, gain-of-function mutations in the \textit{WAS} gene giving rise to a constitutively active protein, were found to cause a distinct pathology, X-linked neutropenia (XLN). XLN is characterized by low neutrophil counts and predisposition to myelodysplasia in the absence of thrombocytopenia and T-cell immunodeficiency\(^7,8\).

The wide spectrum of clinical manifestations highlights the complex role of WASP in various cellular mechanisms.

Clinical manifestations in WAS

Micro-thrombocytopenia

Among clinical manifestations, haemorrhages are frequent (>80% incidence) in WAS and XLT patients, and range from non-life threatening (epistaxis, petechiae, purpura, oral bleeding) to severe
manifestations such as intestinal and intracranial bleeding\textsuperscript{9}. Death of WAS patients is caused, in 21\% of the cases, by haemorrhages\textsuperscript{9,10}. Bleeding is due to severe thrombocytopenia with reduced platelet size, which is the most common finding in WAS and XLT patients (100\% incidence). Thrombocytopenia occurs irrespectively of the severity of the mutation and is possibly caused by instability of mutated WASP in platelets\textsuperscript{11}. Despite intensive research, the mechanisms underlying WASP-related thrombocytopenia and haemorrhages are not completely understood. Megakaryocyte numbers have been reported to be normal in majority of WAS patients\textsuperscript{12-14}, while proplatelet formation depending on actin polymerization and formation of branching structures is conserved when tested in \textit{in vitro} and \textit{ex vivo} cultures\textsuperscript{12}. Peripheral destruction of platelets in the spleen is thought to play an important role in thrombocytopenia, since a substantial correction of the platelet count and size after splenectomy has been reported\textsuperscript{15}. The accelerated destruction could be caused by an intrinsic defect of WASP deficient platelets, showing an increased surface exposure of phosphatidylserine, or could be mediated by autoimmune reaction due to the presence of anti-platelet antibodies reported in patients and in the murine knock out model\textsuperscript{13}, although the latter hypothesis is still a matter of controversy in the field. Finally, defects in filopodia and podosomes could play an additional role in migration of megakaryocytes from the endosteal to the perivascular niche within the bone marrow and during proplatelet formation. Indeed, ectopic release of platelets within the bone marrow of \textit{was}\textsuperscript{+/-} mice has been recently demonstrated\textsuperscript{16}. Overall, these findings indicate that a full comprehension of the mechanisms causing thrombocytopenia still needs to be achieved.

**Eczema in WAS**

The typical skin lesions in WAS and XLT patients resemble acute or chronic eczema in appearance and distribution. Eczema develops in 80\% of the patients\textsuperscript{2,9}, and is heterogeneous in severity and
persistence. Indeed, in its most severe form, eczema is resistant to therapy, persists into adulthood, and facilitates opportunistic skin infections (*Molluscum contagiosum, Herpes simplex*, or bacteria). The incidence and severity of eczema are significantly lower in patients with residual WASP expression\(^2\). The causes of eczema in WAS patients are currently unknown. WAS patients often have elevated IgE levels and develop allergies\(^2\), therefore suggesting an atopic origin. Recently, an imbalance in cytokine production towards the Th2 type has been described in WAS patients’ T cell lines\(^1\), and might contribute to the pathogenesis of eczema and allergy. Abnormal priming of antigen-specific T cells in the skin caused by defective chemotaxis of DCs and Langerhans cells may also play a role\(^1\).

**Autoimmune manifestations**

WAS-associated autoimmune complications are frequently observed. The incidence of autoimmunity in classic WAS is high in the US and European populations (40-72%), while a lower incidence was reported in Japan (22\%)\(^2,9,18\). The most common manifestations are autoimmune haemolytic anemia, cutaneous vasculitis, arthritis and nephropathy. Less common autoimmune manifestations include inflammatory bowel disease, idiopathic thrombocytopenic purpura and neutropenia. Patients frequently suffer from multiple autoimmune manifestation at the same time\(^1\). Development of autoimmunity can have a prognostic value. Indeed, it has been reported that WAS patients who develop autoimmune haemolytic anemia or autoimmune thrombocytopenia early (<180 days) after splenectomy, have a poor prognosis\(^1\). Moreover, autoimmunity is associated with a higher risk of a later development of tumours and an increased risk of mortality\(^9\).

Until now, the mechanisms of WAS-associated autoimmunity have not been clarified. It has been proposed that autoimmunity could be the result of a bystander tissue damage originating from the chronic inflammatory state that is established after incomplete pathogen clearance\(^10\). Another
possible cause is the loss of central or peripheral tolerance to self-antigens. Indeed, several groups including ours have recently described a defective localization and function of naturally occurring CD4+CD25+FOXP3+ regulatory T cells in the absence of WASP19-22.

**Tumours in WAS**

Two distinct surveys report a tumour incidence of 13% and 22%2,9 in WAS patients. Tumours can arise during childhood (especially myelodysplasia), but are more frequent in adolescents and young adults. WAS-associated tumours are mainly lymphoreticular malignancies, with leukaemia, myelodysplasia, and lymphoma (often EBV-positive) resulting in up to 90% of the cases. WAS-associated malignancies have a poor prognosis, since less than 5% of patients survive 2 years after diagnosis9, and result in up to 25% of death cases10. However, current chemotherapy protocols including anti-CD20 monoclonal antibody can result in higher cure rates23,24. Immune deficiency can contribute to the genesis of tumours. Indeed, defective NK cell functions, as well as other alterations of immune surveillance, may play a key role in the susceptibility to tumour development. Additionally, an activating mutation in the Cdc42-binding site of WASP, resulting in X-linked neutropenia, reveals a role of WASP in regulation of cytokinesis and genomic stability in human cells25, leading to the hypothesis that WASP mutations may directly alter cellular homeostasis.

**WAS-XLT clinical scoring system**

The severity of WAS-associated symptoms can be estimated through a scoring system originally developed by Zhu and colleagues26, and slightly refined in subsequent work27. A score of 0.5 or 1, assigned to patients with intermittent or chronic thrombocytopenia and small platelets, and a score of 2, assigned to patients with additional findings of mild, transient eczema or minor infections,
identify XLT patients. Those with treatment-resistant eczema and recurrent infections in spite of optimal treatment receive a score of 3 (mild WAS) or 4 (severe WAS). Regardless of the original score, if a patient develops autoimmune disease or malignancy, a score of 5 is attributed. Score 5A and 5M indicate score 5 with autoimmune disease (A) or malignancy (M), respectively. For a schematic summary of the scoring system, refer to Table I.

**Cellular defects in WAS**

**Hematopoietic stem cells**

Early studies conducted on human samples have demonstrated that WAS RNA is transcribed already at the stage of CD34⁺ hematopoietic stem cells (HSC), and its expression is maintained throughout hematopoietic differentiation. Nonetheless, the cytological appearance of bone marrow of WAS patients is often normal, and CD34⁺ cells isolated from a WAS patient can differentiate into normal numbers of myeloid colonies *in vitro*. Therefore, WASP might be dispensable in early hematopoiesis.

The study of female carriers of a mutated WAS allele shed light on the function of WASP in HSC. Indeed, a non-random X-inactivation in CD34⁺ cells and in mature hematopoietic cells was observed in these individuals, leading to the hypothesis that WASP may play a role in the lyonization process. However, a direct role for WASP in X-chromosome methylation has never been demonstrated. Alternatively, non-random X-inactivation in the bone marrow could be explained by a migratory defect of WASP-null HSC. Indeed, competitive transplantation experiments indicated a role of WASP in the migration of HSC from the fetal liver to the bone marrow, and for their engraftment. However, these results have been significantly challenged, at
least in the murine model, by two reports showing lack of advantage for WASP+ hematopoietic progenitor cells in was+/− female mice32,33. Additionally, in contrast to the skewed pattern observed in WAS female carriers, a random pattern of X inactivation was detected in carriers of XLT34. The information deriving from gene therapy studies will be crucial to define whether human WASP+ HSC show a significant engraftment advantage over WASP-null cells.

**T cell defect in WAS**

T cell defects, hampering both effector and helper functions, are thought to play a crucial role in WAS-associated immunodeficiency. WASP plays a key role in T cell activation and actin cytoskeleton remodelling after the engagement of the T cell receptor (TCR)35-38, and the co-stimulatory molecules CD2839 and CD22. T cell activation is regulated by the formation of the immunological synapse (IS), a polarized cluster of TCR, co-stimulatory molecules, signalling molecules, and integrins at the T cell:APC interface. The IS is a symmetrical structure organized in concentric rings, with the TCR, the TCR-associated molecules, and co-stimulatory molecules residing in the centre, while integrins are localized in the outer ring. Larger molecules such as CD45 and CD43, which may interfere with synapse assembly through steric hindrance, are actively excluded from the IS. To promote their lateral movement on the plasma membrane, the molecules being recruited to the IS are associated with specific cholesterol-enriched membrane microdomains, called lipid rafts. Following TCR engagement, WASP is promptly recruited to the lipid rafts at the IS through WIP41, and Nck42, and is activated in situ by GTP-Cdc4242. In addition, WASP recruitment to the IS could be mediated by CD2 through the adaptor molecules CD2AP and PSTPIP140.

In the absence of WASP, IS can be formed only after strong TCR stimulation43. In addition, lipid raft dynamics during IS formation44, and IS stability45 are compromised. Another level of regulation
of T cell activation is achieved by prompt internalization of the TCR and CD28 co-stimulatory molecule after specific engagement, functions that are defective in WASP deficient cells\textsuperscript{39,46}. As a consequence of impaired signalling through the TCR and co-stimulatory molecules, T cells from WAS patients and \textit{was}\textsuperscript{-/-} mice show defective proliferation as well as impaired secretion of IL-2 and Th1 cytokines\textsuperscript{17,36-38}. These defects are associated with delayed NFAT-1 nuclear translocation and defective T-bet induction\textsuperscript{17,47}. In addition to its role in T cell activation, WASP is also critical for T cell chemotaxis \textit{in vitro} in response to SDF-1\textalpha\textsuperscript{48} and \textit{in vivo} homing to secondary lymphoid organs\textsuperscript{49}. A reduction in the number of circulating naïve CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells may be present in WAS patients, especially at a young age, contributing to the immunodeficiency\textsuperscript{50}. On the other hand, recent studies have highlighted that WASP is dispensable for thymic generation of T cells in mice. Indeed, \textit{was}\textsuperscript{-/-} mice have a relatively normal thymic development, but abrogation of both WASP and N-WASP function through a dominant negative portion of WASP\textsuperscript{51}, or simultaneous knock-out of \textit{N-Was}\textsuperscript{52}, caused the block of thymocyte maturation at the DN3 stage. Thus, N-WASP can complement WASP deficiency to promote the generation of normal numbers of T cells. In addition, the observation that the TCR V\textbeta repertoire is normal in young WAS patients, suggests that WASP absence does not impair thymopoiesis qualitatively\textsuperscript{53}. In the same study, it was observed that the TCR V\textbeta repertoire of WAS patients becomes skewed after 15 years of age\textsuperscript{53}. This finding supports the hypothesis of defective T cell survival in the periphery. Indeed, T lymphocytes isolated from blood of WAS patients are abnormally prone to spontaneous \textit{in vitro} apoptosis due to decreased Bcl-2\textsuperscript{54}, or increased Fas levels\textsuperscript{55}. Despite the above information, the precise relationship between T cell abnormalities and WAS-associated immune deficiency, autoimmunity and cancer remains to be elucidated.

\textbf{Naturally occurring T regulatory cells and the pathogenesis of autoimmunity}
Autoimmunity is a serious and frequent complication in WAS patients, and could be caused by defective peripheral tolerance due to alterations in generation or function of T regulatory cells. Regulatory T cells (Tregs) play a key role in suppressing immune responses and in maintaining immunological homeostasis. Tregs have been shown to prevent autoimmune diseases, to down-modulate immune response to allergens, pathogens and cancer cells, and to mediate peripheral transplantation tolerance\textsuperscript{56,57}. The best characterized subset of Tregs are the CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+} natural Tregs (nTregs), whose differentiation, peripheral survival and function are controlled by TCR engagement, CD28 engagement, FOXP3 and IL-2\textsuperscript{57}. nTregs are generated in the Hassall’s corpuscles within the thymic medulla, where autoreactive T cells may interact with TSLP-producing DCs and acquire a regulatory phenotype, instead of undergoing negative selection\textsuperscript{58}. By this mechanism, nTregs express a broad repertoire of high-affinity TCR, recognizing self antigens, tumor associated antigens and pathogen derived antigens\textsuperscript{57}. The mechanisms by which nTregs are activated and mediate suppression of effector CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are still matter of intense investigation. However, in some experimental settings, they mediate suppression through release of inhibitory cytokines, induction of cytolysis, metabolic interference and modulation of DCs maturation and function\textsuperscript{59}. It is now well established that quantitative and qualitative defects in regulatory T cells may result in the pathology. Indeed, there is evidence that skewing of antigen-specific T cells toward a regulatory instead of a Th1 or Th2 phenotype plays a key role in the maintenance of homeostasis and prevention of autoimmunity and allergy\textsuperscript{60,61}. Therefore, it is possible that defects in nTreg generation and function could be associated with the development of autoimmunity and the unbalanced Th2 response in WAS patients\textsuperscript{17}. To determine whether the absence of WASP is associated with nTreg cell dysfunction, we and others\textsuperscript{19-22} have characterized nTreg cells isolated from was\textsuperscript{-/-} mice and WAS patients. WASP appears to be dispensable for thymic development and steady-state distribution of nTregs to the periphery, although selective advantage for WASP expressing nTreg cells has been reported in competitive settings\textsuperscript{32}. In spite of
a relatively normal number of CD4⁺FOXP3⁺CD25hi cells in spleens of was⁻/⁻ mice and in peripheral blood of patients, nTreg cells show a marked defect in in vitro and in vivo suppressor function. Indeed, in vitro experiments showed that was⁻/⁻ nTregs had a significantly reduced capacity to suppress wt effector T cell proliferation. Following strong TCR stimulation, a residual suppressor activity of was⁻/⁻ nTregs on wt effector T cells could be detected suggesting that the in vitro dysfunction of was⁻/⁻ nTregs is mainly due to an activation defect. Despite the master role of WASP in immune synapse assembly, we showed that, in contrast to effector T cells, murine nTregs failed to polarize F-actin and concomitantly WASP to the site of contact with anti-CD3 mAb-loaded APCs or beads coated with anti-CD3 and anti-CD28 mAbs, therefore, excluding a role of WASP in this process. On the other hand, it cannot be excluded that nTregs assemble a structurally different immunological synapase, or that its assembly follows different kinetics. It is also possible that the activation and functional defects of was⁻/⁻ nTregs are independent from immunological synapse formation and are due to defects in signaling downstream the TCR. Besides defective in vitro nTreg suppression activity in the mouse model and in patients, impaired in vivo suppressive function of was⁻/⁻ nTregs has also been demonstrated. Indeed, was⁻/⁻ nTregs display a defective in vivo suppressor activity towards colitis induced by transfer of wt CD45RBhi cells. In line with that, spontaneous genetic reversion of WAS mutations in nTregs of a WAS patient correlated with amelioration of autoimmunity. In addition to an intrinsic dysfunction in mediating suppression, WASP-deficient nTregs may also display impaired migration, survival and/or proliferation in vivo. Indeed, we demonstrated that was⁻/⁻ nTregs transferred in wt recipients were unable to reach lymph nodes draining the site of OVA immunization and thus modulate an in vivo response to OVA. Consistently, WASP expression confers selective advantage to nTregs, in both the was⁻/⁻ murine model and in a revertant patient. The selective advantage for nTregs appeared to be stronger than that occurring in naïve T lymphocytes. Given the key role played by these cells in maintaining
peripheral tolerance, all these findings suggest that nTreg dysfunction might participate in the high susceptibility of WAS patients to developing allergies and multiple autoimmune disorders.

**Dendritic cells in WAS**

The complex immunodeficiency observed in WAS is partially due to defective cellular trafficking and impaired antigen uptake. The effects of WAS gene mutation on dendritic cells, which are antigen presenting cells, result in severe alterations in migration, antigen presentation, cell adhesion and T cell priming. Indeed, the ability of DCs to migrate through tissues and endothelial vessels depends on complex mechanisms involving reorganization of the actin cytoskeleton in response to different stimuli. Since WASP has a crucial role in controlling actin architecture and rearrangement, many defects in macrophages and dendritic cells have been reported. In fact, the initial response to a stimulus consists of cellular polarization, lamellipodia and filopodia formation and subsequent adhesion. In addition, DCs assemble podosomes, special adhesion structures showing a unique organization with actin filaments forming foci containing different elements, such as the actin nucleating factor Arp2/3 complex, and WASP surrounded by a ring of integrins and integrin associated proteins. Many groups have investigated the role of WASP in the formation of podosomes in monocyte-derived DCs, in macrophages and osteoclasts. Analysis of these cells in WAS patients reveal a dramatic lack of podosomes. Interestingly, podosome defect results in alteration of β2 integrin localization, which remain dispersed with consequent decreased adhesion to ICAM-1, a ligand for β2 integrin. These findings suggest that WASP plays a crucial role in providing a platform for integrin organization at the cell membrane. Moreover, was deficient dendritic cells and macrophages fail to form leading lamellipodia resulting in a defective chemotactic response to different chemoattractants, such as FMLP, MCP-1 and macrophage inflammatory protein-1a. In particular, the migratory response of was mutant DCs toward the CCR7 ligands,
CCL21 and CCL19, which are highly expressed on high endothelial venules and lymphatic endothelium, is severely decreased\(^{68,69}\). Similarly, chemotaxis of \(\text{was}^+\) deficient immature DCs to CCL3, important for mobilization to inflammatory sites, is significantly reduced compared to controls\(^{69}\). Additionally, \(\text{was}^-\) DCs do not spread normally and fail to form persistent leading edge, resulting in defective directional migration and inefficient homing of \(\text{was}^-\) DCs into draining lymph nodes\(^{70}\). Finally, recent reports describe that in addition to the impaired motility, \(\text{was}^-\) DCs show defective interaction with and activation of T cells in lymph nodes\(^{70,71}\). Indeed, DCs from \(\text{was}^-\) deficient mice are impaired in presenting antigens to naïve CD8\(^\pm\) T cells following immunization with DEC205, especially at low doses\(^{71}\). These data highlight the relevance of WASP in dendritic cells during the first phases of adaptive immune response and together with altered migration of \(\text{was}^-\) DC could contribute to the abnormal function of these cells.

**Role of WASP in B cells**

The role of WASP in B cells has not been explored in detail until recent years. Initially, reports carried out primarily on \(\text{was}^-\) murine models did not evidence defects in terms of development, number and functionality of WASP-deficient B cells\(^{37,38}\). Nonetheless, an intrinsic B cell defect appears evident due to the abnormal distribution of serum immunoglobulin classes and the inability of severe WAS patients to respond to T-independent antigens, such as polysaccharides\(^1,72\), for which T cell help in triggering immune response is not required.

In contrast to that observed in T lymphocytes, activation of WASP-deficient B lymphocytes can occur normally. Indeed, measurement of calcium fluxes after BCR engagement highlighted a mild defect in WASP-deficient EBV-transformed B cell lines\(^{73}\), but not in primary B cells isolated from WAS patients\(^{74}\). Moreover, B cells isolated from \(\text{was}^-\) mice did not show any activation defect\(^{37,38}\).
The description of B cell anomalies was mainly focused on the defective cytoskeletal-dependant processes of WASP-deficient B cells, leading to decreased migratory ability, adhesion and formation of long protrusions\textsuperscript{72,75}. Additionally, defective cytoskeletal reorganization has been shown in EBV-transformed B cell lines from WAS patients\textsuperscript{76}. Consequently, the decreased motility of WASP-deficient B cells has been considered the main cause of findings showing diminished B cell number in peripheral blood and secondary lymphoid organs of WAS patients\textsuperscript{50,77} and later confirmed in the \textit{was}\textsuperscript{-/-} murine model\textsuperscript{72}.

Recently, reports from Remold-O’Donnell’s group on a large number of WAS patients revealed an early deficit of B cells starting from infancy and indicating a defective cellular output\textsuperscript{50}. Their subsequent characterization of B cell defect in WAS patients led to identification of a phenotypic perturbation with regard to complement receptors and CD27\textsuperscript{78}. In fact, WAS patients’ B cells show a markedly reduced expression of CD21/CD35 receptors, that may be responsible for impaired ability of antigen capture and presentation. Additionally, in comparison with age-matched normal controls, WAS patients present a reduced amount of CD27\textsuperscript{+} post-germinal center B cells, indicating a defective differentiation, despite normal amount of class switching\textsuperscript{78}.

The underlying causes of these alterations are unknown, but recently two reports performed in \textit{was}\textsuperscript{-/-} mice have clearly indicated a role for WASP in peripheral homeostasis of mature B cell subsets\textsuperscript{32,33}. In fact, WASP seems to be dispensable for early B cell development, while its deficiency is detrimental for completion of B cell maturation, starting from transitional stage and affecting in particular splenic marginal zone (MZ) and peritoneal B1a cells. This phenotype appears to be caused by a defective homeostasis and/or retention of mature B cells, rather than increased apoptosis of WASP-deficient B lymphocytes\textsuperscript{32,33}.

Importantly, B cell involvement in the pathogenesis of autoimmune manifestations in WAS still needs to be addressed. A recent European study on the long-term outcome of BMT in WAS patients indicated mixed/split lymphocyte chimerism as a strong risk factor to develop autoimmunity\textsuperscript{79}. In
fact, in WAS patients the development of autoimmune diseases is found to be associated with high levels of circulating IgM\textsuperscript{18}. Moreover, Humblet-Baron and colleagues have recently shown that was\textsuperscript{-/-} mice have circulating autoantibodies against single strand DNA (ssDNA) and double strand DNA (dsDNA), providing the first evidence of an alteration of B cell tolerance in was\textsuperscript{-/-} mice\textsuperscript{20}. These alterations were confirmed in our unpublished observations in was\textsuperscript{-/-} mice where we found an increased amount of serum immunoglobulins, in particular of IgG2a, a subclass that plays a critical role in the pathogenesis of humoral autoimmunity (M.B., manuscript in preparation).

Decreased complement receptor expression is intriguing evidence as it could predispose WAS patients to developing autoimmune manifestations\textsuperscript{78}. Several lines of evidence point toward the involvement of reduced CD21/CD35 in induction of autoimmunity. In fact, CD21/CD35 expression is decreased in B cells of patients with systemic lupus erythematosus (SLE)\textsuperscript{80} and also in the murine model of SLE prior the development of clinical disease, suggesting a role of this alteration in break of self-tolerance\textsuperscript{81,82}.

In conclusion, further comprehension of the intrinsic defects of was\textsuperscript{-/-} B cells appears to be crucial to fully understand the pathophysiology of WAS and to develop more efficacious therapies.

**Current therapeutic approaches and perspectives for gene therapy in WAS**

Currently, the only curative therapeutic option for WAS patients is hematopoietic stem cell transplantation (HSCT). When a related HLA-identical donor (RID) is available, HSCT leads to over 80% survival rate\textsuperscript{83-86}. On the other hand, transplantation using the bone marrow of a mismatched related donor (MMRD) results in a decreased survival rate\textsuperscript{83-86}. In addition, this type of transplant is associated with an elevated risk of developing life-threatening EBV\textsuperscript{+} lymphoproliferative syndrome, infections, autoimmunity and graft-versus-host disease (GVHD)\textsuperscript{84}. 
When a suitable related donor is not available, bone marrow or cord blood transplantation from a matched unrelated donor (MUD) is a valid therapeutic option, leading to 71%-81% of survival rate\textsuperscript{23,84,85}. A better outcome for MUD and MMRD HSCT in patients younger than 5 and 2 years of age, respectively, has been reported suggesting that transplants should be performed early in life\textsuperscript{79,84,85}. Successful HSCT with establishment of full chimerism leads to restoration of immune and hemostatic functions. In patients with stable mixed chimerism in both myeloid and lymphoid compartments clinical conditions usually improve\textsuperscript{23,87}. In spite of advances in HSCT, patients lacking a matched donor still need the development of alternative approaches. Therefore, the implementation of new therapeutic strategies, such as transplantation of autologous gene corrected hematopoietic stem cells, is highly desirable because it will avoid rejection and GVHD and could be applicable to all WAS patients lacking a suitable bone marrow donor, allowing the timely treatment of the disease. The rationale for gene therapy is also supported by the observation of frequent spontaneous somatic revertants conferring selective advantage to WASP-expressing cells\textsuperscript{88}. At present, retroviral vectors based on the murine Moloney leukaemia virus (MMLV) have been used for the treatment of patients with SCID-X1, ADA-SCID and X-CGD\textsuperscript{89-91}. \textit{In vitro} studies on human WASP deficient B and T cell lines transduced with oncoretroviral vectors have demonstrated the restoration of proliferative response to anti-CD3 and cytoskeleton remodelling\textsuperscript{92-94}. Moreover, the availability of \textit{was}\textsuperscript{-/-} mice has allowed assessing the efficacy of WAS gene therapy \textit{in vivo}. In early studies, \textit{was}\textsuperscript{-/-} bone marrow cells were transduced with murine WASP-encoding retroviral vectors, and injected into lethally irradiated \textit{rag2}\textsuperscript{-/-} or \textit{was}\textsuperscript{-/-} recipient mice\textsuperscript{94,95}. Following successful engraftment and multilineage differentiation of transduced cells, TCR-driven T cell proliferation as well as cytokine production were improved\textsuperscript{95,96}. Klein and co-workers demonstrated an attenuation of the colitogenic potential of \textit{was}\textsuperscript{-/-} T cells\textsuperscript{95}, and Strom and colleagues reported the normalization of a secondary immune response to influenza virus after gene therapy\textsuperscript{96}. Very recently, a gene therapy trial for WAS was initiated in Germany using an MLV derived retroviral vector encoding
the full WASp cDNA. Preliminary data from the first two patients eighteen months after gene therapy, indicate amelioration of the clinical phenotype with correction of thrombocytopenia and resolution of eczema and autoimmunity (Boztug et al, 2008, Clinical Experimental Immunology 154, VIII ESID meeting). The use of MLV vector in the context of WAS raises several concerns in view of the adverse events that occurred in X-SCID and X-CGD gene therapy trials. Since lentiviral vectors have been demonstrated to be less genotoxic than retroviral vectors, we and others have developed a gene transfer approach based on a lentiviral vector encoding the human WASP cDNA under the control of the human WAS endogenous promoter. We have demonstrated that our lentiviral vector encoding a 1.6 kb fragment of the human WAS endogenous promoter is able to successfully restore WASP expression in CD34+ HSCs, T cells, B cells, and DCs, and to correct TCR-driven activation in T cell lines derived from WAS patients. Additionally, in vivo studies performed in two different was−/− strains showed evidence of long-term multilineage WASP expression in hematopoietic cells and correction of T and B cell functions. Importantly, long-term observation of a large cohort of gene therapy treated mice did not display any severe adverse event related to vector toxicity. All these findings represent a critical step in moving towards the implementation of a lentiviral vector-mediated gene therapy clinical trial.

Concluding remarks

Wiskott-Aldrich syndrome is a severe immunodeficiency with a characteristic variable clinical phenotype. The molecular bases of this primary immunodeficiency have been extensively explored, and many cellular defects have been reported as responsible of the immunological phenotype, whereas pathogenesis of autoimmunity, malignancies and thrombocytopenia still remain to be fully understood. Nonetheless, the recent advances in the comprehension of molecular and cellular
mechanisms have been crucial, not only in understanding the biology of this syndrome, but also in providing new and efficacious therapeutic tools.
Authorship

Contribution: MB, FM, AA, AV and MGR wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
Acknowledgements

The work of the authors in the field of gene therapy for Wiskott-Aldrich syndrome is supported by grants from the Italian Telethon Foundation (to AA, AV and MGR) and from EU CONSERT (to MGR, AA). The research was partially funded by Network Operativo per la Biomedicina di Eccellenza in Lombardia (NOBEL to MGR and AV), Fondo Italiano per la Ricerca di Base (FIRB, to MGR) and Ministero della Salute RF 2007 Giovani Ricercatori Grant to MB.
References


**Table I:** WAS scoring system according to Zhu and colleagues, with subsequent refinements. Adapted with permission from table published by Imai K, Nonoyama S, Ochs HD. WASP (Wiskott-Aldrich syndrome protein) gene mutations and phenotype. *Curr Opin Allergy Clin Immunol.* 2003; 3: 427-436. © Wolters Kluwer Health.

<table>
<thead>
<tr>
<th>Clinical scores</th>
<th>XLT</th>
<th>WAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Eczema</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Immunodeficiency</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Autoimmunity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malignancy</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Recent advances in understanding the pathophysiology of Wiskott-Aldrich syndrome

Marita Bosticardo, Francesco Marangoni, Alessandro Aiuti, Anna Villa and Maria Grazia Roncarolo