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Characterization of invariant NKT cell phenotype and function in Wiskott-Aldrich Syndrome (WAS)

Nome e Cognome del dottorando

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To Rodrigo: my energy, my love, my life.

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1. ABSTRACT

WAS protein (WASp) is a key regulator of actin cytoskeleton in hematopoietic cells. Mutations of WAS gene cause the Wiskott-Aldrich Syndrome (WAS). Although WASp is involved in various immune cell functions, its role in invariant NKT cells (iNKT) has never been investigated. Defects of iNKT cells could contribute to the pathogenesis of several WAS features, such as recurrent infections and high tumor incidence. Indeed, we found a profound reduction of circulating iNKT cells in WAS patients, directly correlating with the severity of clinical phenotype**.** To better characterize iNKT cell defect in the absence of WASp, we analyzed *was*$^{-/-}$ mice. iNKT cell number is significantly reduced in thymus and periphery of *was*$^{-/-}$ mice as compared to wt controls. Moreover analysis of *was*$^{-/-}$ iNKT cell maturation reveals a complete arrest at the CD44^{+}NK1.1^{-} intermediate stage. Notably, generation of BM chimeras demonstrated a *was*$^{-/-}$ iNKT cell autonomous developmental defect. The lack of WASp does not affect IL-15 signaling, which is important in iNKT cell development. Conversely WASp is required for the control of homeostatic proliferation. *was*$^{-/-}$ iNKT cells are also functionally impaired, as suggested by the lower expansion and reduced secretion of IL-4 and IFN-γ upon *in vivo* activation. Furthermore, *in vitro* assays suggest that the functional defect of WASp null iNKT cells is TCR-mediated and indicated that the defective IL-4 production is due to a *was*$^{-/-}$ iNKT cell autonomous defect, whereas the lower IFN-γ production is caused by an inefficient crosstalk between *was*$^{-/-}$ iNKT cells and *was*$^{-/-}$ DCs. Altogether, these results demonstrate the relevance of WASp in integrating signals critical for development and functional differentiation of iNKT cells, and suggest that defects in these cells may play a role in WAS pathology.

1. ITALIAN ABSTRACT

WASp è una proteina che regola il rimodellamento dell'actina del citoscheletro nelle cellule ematopoietiche. Mutazioni nel gene che codifica per WASp (*WAS*) causano la Sindrome di Wiskott-Aldrich (WAS). Sebbene WASp sia coinvolto in svariate funzioni delle cellule del sistema immunitario, il suo ruolo nei linfociti invarianti NKT (iNKT) non è mai stato investigato. Difetti delle cellule iNKT potrebbero infatti contribuire allo sviluppo di alcune caratteristiche dei pazienti WAS quali le infezioni ricorrenti e l'alta incidenza tumorale. Infatti il nostro studio ha rivelato una profonda riduzione numerica dei linfociti iNKT periferici nei pazienti WAS, direttamente correlata con la severità del fenotipo clinico dei pazienti. Per definire ulteriormente il fenotipo delle cellule iNKT prive di WASp, abbiamo esteso l'analisi ai topi *was*$^{-/-}$. Le cellule iNKT sono significativamente ridotte anche nel timo e negli organi linfoidi periferici dei topi *was*$^{-/-}$ rispetto ai controlli wt. Inoltre l'analisi dello sviluppo delle cellule *was*$^{-/-}$ iNKT cell ha messo in luce un completo blocco maturativo allo stadio intermedio CD44^{+}NK1.1^{-}. In particolare, la generazione di chimere di midollo osseo, ha dimostrato un difetto maturativo intrinseco delle cellule *was*$^{-/-}$ iNKT. L'assenza di WASp non altera la stimolazione indotta dall'IL-15, che è importante nello sviluppo delle cellule iNKT. Contrariamente, WASp è coinvolto nel controllo della proliferazione omeostatica di questa tipologia cellulare. Le cellule iNKT prive di WASp presentano anche un difetto funzionale, come messo in evidenza dalla ridotta secrezione di IL-4 e IFN-γ dopo la loro attivazione *in vivo*. In aggiunta, saggi funzionali condotti *in vitro*, suggeriscono che il difetto funzionale delle cellule iNKT prive di WASp sia mediato dal TCR e che la ridotta produzione di IL-4 sia causata da un difetto funzionale intrinseco alle cellule iNKT, mentre la minor produzione di IFN-γ sembra derivare da un'interazione non efficiente tra le cellule *was*$^{-/-}$ iNKT cells e le cellule dendritiche *was*$^{-/-}$. Nel loro insieme questi risultati dimostrano il ruolo rilevante di WASp nell'integrare segnali critici per lo sviluppo e la funzione delle cellule iNKT, e suggeriscono che i difetti di questa popolazione linfocitaria possano contribuire alla patologia della Sindrome di Wiskott-Aldrich.

2. INTRODUCTION

2.1 THE WISKOTT-ALDRICH SYNDROME

In 1937, Alfred Wiskott, a German paediatrician, described a "familial and innate thrombopathia" affecting three brothers. The pathology was characterized by thrombocytopenia, bloody diarrhea, eczema, recurrent ear infections, and eventually led to the death of the patients because of intestinal hemorrhages and sepsis (1). In 1954, Aldrich and colleagues demonstrated the X-linked inheritance of this syndrome by analyzing the pedigree of a family in which 16 out of 40 males -but no females- were affected (2).

Nowadays, Wiskott-Aldrich Syndrome (WAS, OMIM 301000) is known as a complex and severe X-linked disorder characterized by micro-thrombocytopenia, eczema, immunodeficiency and increased risk to develop autoimmunity and lymphomas. WAS affects 1 to 10 out of a million male newborns (3), whose life expectancy is about 15 years (4).

The identification of the gene whose function is lost in WAS (*WAS* gene) has been achieved In 1994 through a positional cloning strategy (5). The protein encoded by the *WAS* gene (Wiskott-Aldrich Syndrome protein, or WASp) is a hematopoietic specific (6) regulator of actin nucleation in response to signals arising at the cell membrane (7).

The discovery of the *WAS* gene has allowed to link other diseases to alterations in this locus. Mutations impairing but not abolishing WASp expression, can cause X-linked thrombocytopenia (XLT). This disease can be chronic (8) or intermittent (9), 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 *WAS* gene giving rise to a constitutively active protein, were found to cause a distinct pathology, X-linked neutropenia (XLN). It is characterized by low neutrophil counts and predisposition to myelodysplasia in the absence of thrombocytopenia and T-cell immunodeficiency (10, 11).

2.2. CLINICAL MANIFESTATIONS OF WAS

2.2.1. Hemorrhages

Hemorrhages are frequent (>80% incidence) in WAS patients, and range from non-life threatening (epistaxis, petechiae, purpura, oral bleeding) to severe manifestations such as intestine and intracranial bleeding. Death of WAS patients is caused, in 25% of the cases, by hemorrhages (12). Bleeding is due to severe thrombocytopenia (ranging from 6000 to 70000 per µl (4)) with reduced platelet size. This is the most common finding in WAS and XLT patients (100% incidence) and is due to the lack of WASp in platelets irrespectively of the severity of the mutation, possibly as a consequence of instability of the mutated protein (13).

Despite intensive research, the mechanisms underlying WASp-related thrombocytopenia and hemorrhages are incompletely understood. Indeed, defective thrombopoiesis, accelerated platelet destruction, and platelet dysfunction have been hypothesized, but their relationship with WASp deficiency is unclear.

Whether defective thrombopoiesis contributes to thrombocytopenia in WAS patients is controversial. Indeed, the number and cytological appearance of megakaryocytes in the bone marrow of XLT and WAS patients can be normal (4, 8, 14). On the other hand, defective platelet production has been suggested based on accelerated platelet turnover in WAS patients (14), and decreased *in vitro* proplatelet formation by megakaryocytes derived from WAS patients (15). These latter results were challenged by another study demonstrating that *in vitro* proplatelet formation by megakaryocytes derived from WAS patients was normal (16). An alternative hypothesis reconciling normal thrombopoiesis with decreased platelet release in the blood has recently proposed. Indeed, *in vivo* studies performed in *was*$^{-/-}$ mice showed that ectopic platelet shedding within the bone marrow can occur, hampering the platelet release in the peripheral blood despite normal platelet production by megakaryocytes (17). These studies, however, await confirmation on bone marrow specimens isolated from WAS patients.

There is more general agreement on a role of peripheral platelet destruction in the pathogenesis of WAS-related thrombocytopenia. Indeed, decreased half-life of circulating platelets is a common finding in WAS patients (14, 18, 19), and can be due

to intrinsic platelet abnormalities (20) or to autoantibodies (4, 21, 22), finally leading to splenic sequestration (20). Accordingly, splenectomy can correct both platelet numbers and size (21, 23), but it is usually discouraged since it exposes the patient to a high risk of sepsis (23). Moreover, some splenectomised WAS patients suffer a thrombocytopenia relapse, which is usually immune-mediated (21) and predicts a severe prognosis (24).

Platelet dysfunction can also contribute to the hemorrhages frequently observed in WAS patients. Indeed, patients having normal number of platelets after splenectomy are not completely protected from bleeding episodes (23). The mechanisms proposed are defective platelet activation after stimulation with thrombin (22), and defective platelet adhesion to fibrinogen through the αIIbβIII integrin (25).

2.2.2. Eczema

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 (4, 26), 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). Severity of eczema is significantly lower in patients with residual WASp expression (4).

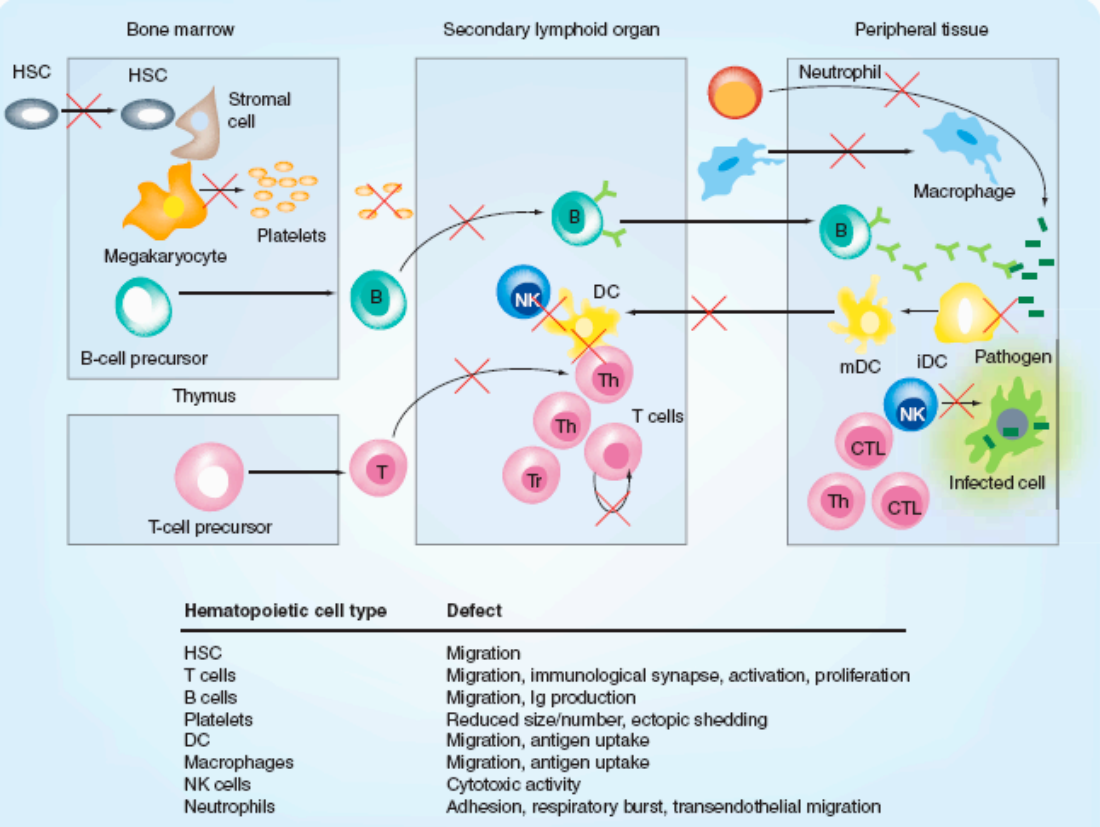
The causes of eczema in WAS patients are currently unknown. WAS patients often have elevated IgE levels (4), therefore suggesting an atopic origin. Indeed, WAS patients often develop allergy (4). Recently, an imbalance in cytokine production towards the Th2 type has been described in WAS patients' T cell lines (27), 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 (3).

2.2.3. Immunodeficiency

WAS patients have often symptomatic infections caused by various microorganisms including bacteria, viruses and fungi. Frequency and severity of infections are higher in WASp-negative patients, as compared to WAS patients with residual WASp

expression (4), and cause nearly 50% of death cases (12). Most common bacterial infections affect the respiratory tract, the ear, the gut, the skin, and the urinary tract. Systemic infections, such as meningitis and sepsis, are also reported (4). Bacterial infections are mainly due to encapsulated bacteria, probably because of the inability to produce antibodies against polysaccharide antigens (14). Viral infections are mostly due to *Herpes simplex* (can be severe and disseminated) and to *Cytomegalovirus* spp. (encephalitis and hepatitis). WAS patients also develop *Molluscum contagiosum* and *Papillomavirus* infections. Fungal infections due to *Candida* spp. and *Aspergillus* spp. as well as opportunistic pulmonary infections due to *Pneumocystis carinii* are also frequent in these patients (4).

The complex immunodeficiency caused by the lack of WASp results from the dysfunction of many immune cell types. Indeed, absence of WASp impairs T cell activation (27-29), NK lytic activity (30, 31), B cell function (32), phagocytosis (33, 34) and antigen presentation by DCs (35), and microbial killing by neutrophils (36). In addition, migration of WASp-deficient hematopoietic stem cells (37) and leukocytes (38) is globally defective. Finally, WAS patients, especially young ones, can suffer from T and B lymphopenia (39). For a schematic summary of the reported cellular defects refer to Figure I.



Hematopoietic cell type	Defect
HSC	Migration
T cells	Migration, immunological synapse, activation, proliferation
B cells	Migration, Ig production
Platelets	Reduced size/number, ectopic shedding
DC	Migration, antigen uptake
Macrophages	Migration, antigen uptake
NK cells	Cytotoxic activity
Neutrophils	Adhesion, respiratory burst, transendothelial migration

Figure I: Cellular functions altered by WASp deficiency.
Abbreviations used: HSC: hematopoietic stem cell; B: B lymphocyte; NK: natural killer lymphocyte; T: T lymphocyte; Th: T helper lymphocyte; CTL: cytotoxic T lymphocyte; iDC: immature dendritic cell; mDC: mature dendritic cell. Modified from Trifari et al (40).

2.2.4. Autoimmunity

WAS-associated autoimmune complications are frequently observed. They have been reported to affect from 25% to 72% of the patients (4, 24, 26), irrespectively of WASp expression and overall disease severity (4). The most common manifestations are autoimmune hemolytic anemia, cutaneous vasculitis, arthritis and nephropathy. Less common autoimmune manifestations include inflammatory bowel disease, idiopathic purpura thrombocytopenia and neutropenia. Patients frequently suffer from multiple autoimmune manifestations at the same time (24).

Development of autoimmunity can have a prognostic value. Indeed, it has been reported that WAS patients who develop autoimmune hemolytic anemia or autoimmune thrombocytopenia early (<180 days) after splenectomy, have a poor prognosis (24). Moreover, autoimmunity is associated with a higher risk of a later

development of tumors and with an increased risk of mortality (26).

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 (12). Another possible cause of autoimmunity is the loss of central or peripheral tolerance to self-antigens. Given the role of WASp in TCR signaling, it is possible that WASp deficiency impairs negative selection of thymocytes, leading to recirculation of abnormal proportions of potentially self-reactive T cells. It is also possible that WASp absence impairs localization or function of regulatory T cells, which can no longer suppress the activation of autoreactive T cells in the periphery. 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 WASp (41-44).

2.2.5. Tumors

Two distinct surveys report a tumor incidence of 13% and 22% (4, 26) in WAS patients. Tumors can arise during childhood (especially myelodysplasia) but are more frequent in adolescents and young adults. WAS-associated tumors are mainly lymphoreticular malignancies, since leukemia, myelodysplasia, and lymphoma (often EBV-positive) cover 90% of the cases. WAS-associated malignancies have a poor prognosis, as less than 5% of patients survive 2 years after diagnosis (26), and cover up to the 25% of the death cases (12).

The observation that a consistent proportion of malignancies is associated to EBV infection, leads to the hypothesis that immune deficiency can contribute to the genesis of tumors. Defective NK cell functions, as well as other alterations of immune surveillance, may play a key role in the susceptibility to tumor development. However, other mechanisms can be involved, since the highest lymphoma incidence (44%) can be conferred by a single splice site mutation that is otherwise associated with a mild clinical phenotype (45). To this regard, it has been recently reported that WASp regulates cytokinesis and genomic stability in human cells (46), leading to the hypothesis that WASp mutations may directly alter cellular homeostasis.

2.2.6. Scoring system

The severity of WAS-associated symptoms can be estimated through a scoring system originally developed by Zhu and colleagues (47), and slightly refined in subsequent works (4, 9).

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. For a schematic summary of the scoring system, refer to Table I.

	XLT			WAS		
Clinical scores	0.5	1	2	3	4	5
Thrombocytopenia	+/-	+	+	+	+	+
Eczema	-	-	+/-	+	++	++/-
Immunodeficiency	-	-	+/-	+	++	++/-
Autoimmunity or tumors	-	-	-	-	-	+

Table I: WAS scoring system according to Zhu and colleagues (47), with subsequent refinements (4, 9).

2.3. MOLECULAR DEFECT CAUSING WAS

2.3.1. Identification and characterization of the gene

By taking advantage of a previous mapping study which localized the *WAS* gene to the region Xp11.22-Xp11.3 (48), Derry and colleagues isolated the *WAS* gene by positional cloning and demonstrated mutations in lymphoblastoid cell lines derived from patients with WAS or XLT (5). The *WAS* gene encompasses 12 exons, and encodes a 502-aminoacid intracellular protein (WASp). WASp is expressed in all non-erythroid hematopoietic cells (6, 13).

The mouse *was* gene was identified in parallel to the human *WAS* gene. The mouse *was* gene resides on the X chromosome, and encodes a 520-aminoacid protein which shares 86% identity with the human counterpart (5). Two *was* knock-out ($was^{-/-}$) mouse strains were constructed by disruption of the *was* gene by deletion of exons 4 to 11 (49), or by a large insertion in exon 7 (50). In both cases WASp expression was completely abrogated. These mouse strains have been validated as relevant models of WAS, since they carry many of the cellular defects originally described in WAS patients (49, 50), and are immunodeficient (51).

2.3.2. Molecular functions of WASp

WASp is the founding member of a family of proteins whose function is to integrate the signals from many input pathways to promote actin cytoskeleton remodeling. The other members of the WASp family in mammals are the neural-WAS protein (N-WASP) and the scar/WAVE proteins (WAVE1, WAVE2, and WAVE3) (52) (Figure IIA). All these proteins share a highly similar C-terminal portion composed by a polyproline-rich region (PPT), and a Verprolin/Cofilin/Acidic (VCA) domain. In particular, the VCA domain can bind and activate the Arp2/3 complex, one of the most powerful activators of nucleation of new F-actin filaments stemming from pre-existing ones ("branching" process). On the other hand, the N-terminal portion of the WASp family proteins, which actually controls their activation, is less conserved. Indeed, WASp and N-WASp contain an EVH1 (Ena/VASP homology 1) domain, a basic region (BR), a CDC42 and Rac interactive binding domain (CRIB), while the

WAVE proteins lack such a structure, retaining the basic region only. WASp and N-WASp have a very similar domain structure, but differ for the expression pattern, since the former is hematopoietic-specific and the latter is ubiquitous, but more abundant in the nervous system. The very high structural similarities between WASp and N-WASp have led to the speculation that the molecular activation processes, as well as the binding partners, demonstrated for either of the two proteins, are actually relevant for both. This is illustrated by the recent demonstration of the redundancy of WASp and N-WASp for T cell differentiation (53). For a schematic summary of proteins interacting with WASp, see Figure IIB.

Constitutive interaction between the WASp EVH1 domain and the WASp interacting protein (WIP) stabilizes WASp through inhibition of calpain and proteasome degradation (54-56). Moreover, it could also inhibit spontaneous WASp activation (demonstrated experimentally for N-WASp (57)). It has been hypothesized that the dissociation of the WIP/WASp complex is key for WASp activation in T cells stimulated through the TCR (58). However, evidences confuting this hypothesis have been reported very recently. Indeed, Dong and colleagues demonstrated that a WIP/WASp fusion protein is functional in TCR-stimulated T cells (59).

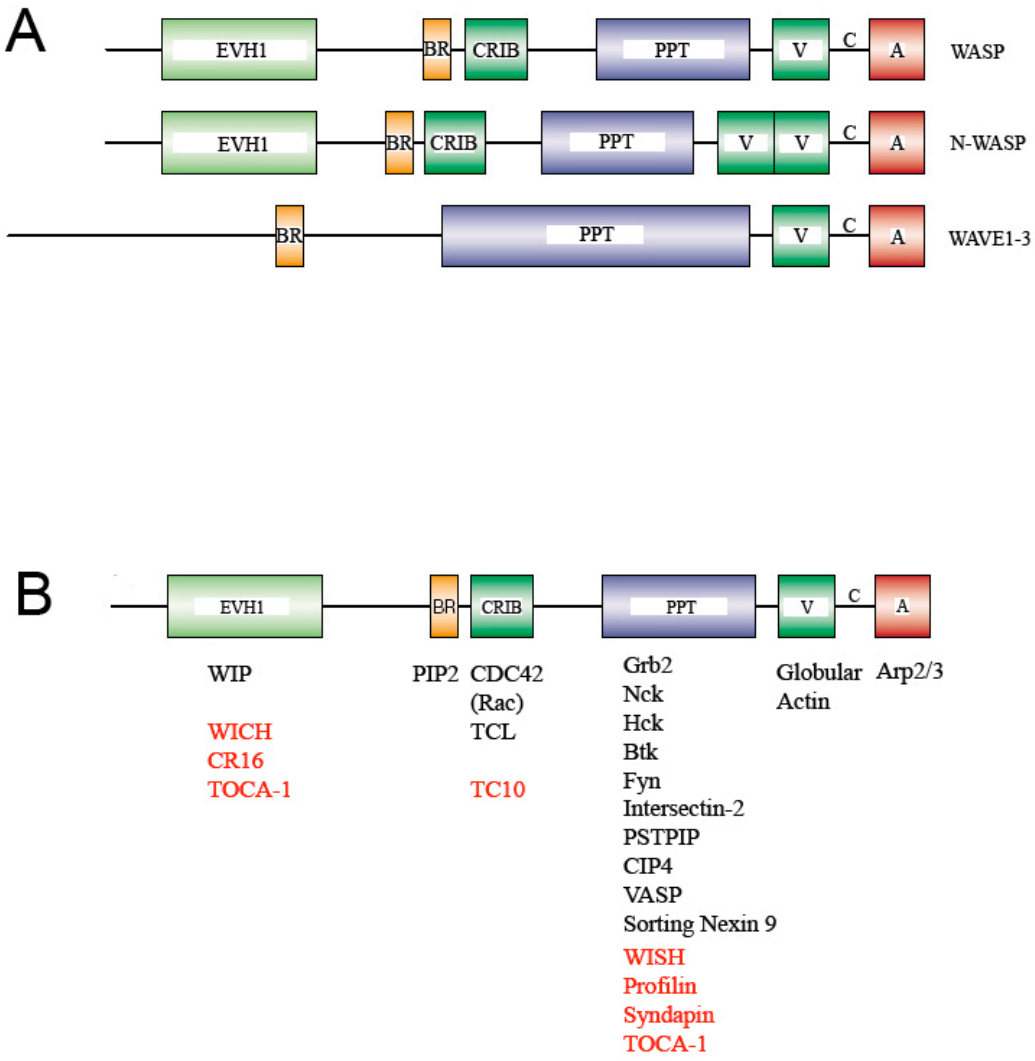


Figure II: Structure of WASp family proteins.
A. Schematic representation of the WASp family proteins in mammals. **B.** Proteins interacting with WASp. The interactions shown experimentally for N-WASp are reported in red. Modified from Thrasher et al (60).

The actin-remodeling activity of WASp is mediated by the C-terminal VCA domain, which directly binds and activates the actin nucleation complex Arp2/3. In resting conditions, WASp assumes an auto-inhibited conformation characterized by the folding of the VCA domain on the CRIB domain through hydrophobic interactions (61). As a consequence, the VCA domain of WASp is unable to bind the Arp2/3 complex and promotes actin nucleation. The molecular activation of WASp can be achieved by cooperative binding of multiple interactors. Binding of phosphatidylinositol (4,5) bi-phosphate (PIP2) to the N-terminal basic region of WASP can synergize with the binding of GTP-loaded CDC42 to the CRIB domain (62), or the binding of SH3 domain containing proteins (such as Grb2, Nck) to the PPT region (63), to release auto-inhibition and promote actin nucleation. Binding of other SH3 domain containing proteins to WASp PPT region mediates a number of other important functions. Indeed, interaction with TOCA-1 amplifies the activatory signal mediated by PIP2 and GTP-CDC42 (shown experimentally for N-WASp (64)); the Intersectin-2 (65) and Sorting Nexin 9 (66) can recruit WASp to endocytotic vesicles; binding of CIP4 could mediate interaction with the microtubule cytoskeleton (67); interaction with VASP could facilitate filopodia formation (68). In addition, several kinases and phosphatases can interact with WASp SH3 domain, and regulate its activation through phosphorylation and dephosphorylation of a single tyrosine residue (Y291). Phosphorylation of Y291 (Y293 in murine WASp), achieved by Fyn (in T cells) (69), Btk (in B cells) (70), and Hck (in phagocytes) (71), increases the basal activity of WASp conferring sensitivity to input signals through SH2-domain containing proteins (72), and leading to cell activation (69). In some circumstances tyrosine phosphorylation may occur independently of binding to GTP-bound CDC42 (69, 71). In addition, a recent finding underlines the absolute requirement for WASp phosphorilation during multiple cellular task, including migration, phagocytosis and proliferation (73). Dephosphorylation of Y291 is achieved by the PTP-PEST phosphatase, which is recruited to WASp through the adaptor protein PSTPIP (69). Moreover, constitutive phosphorylation at residues S483 and S484 by CK2 kinase is required for optimal activation of the Arp2/3 complex (74). A simplified model for WASp activation is depicted in Figure III.

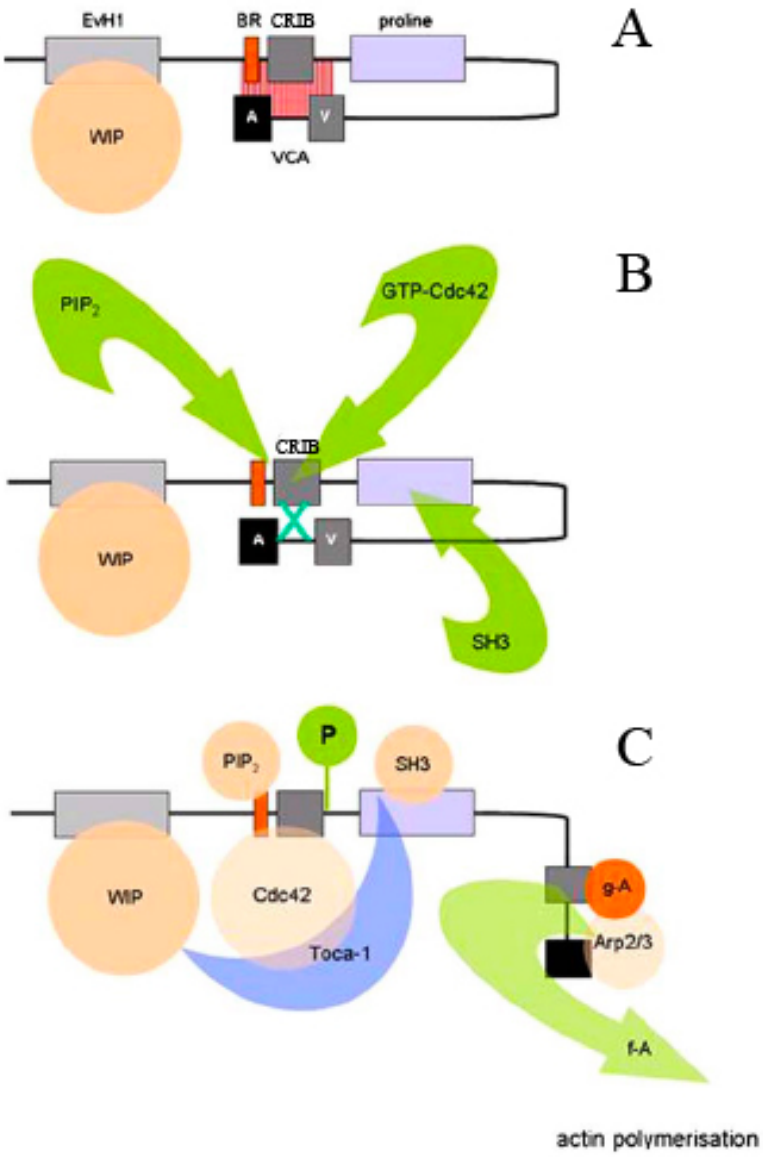


Figure III: Model depicting the mechanism of WASp activation.
A. Autoinhibited conformation by hydrophobic interactions between the CRIB domain and the VCA domain. **B.** By cooperative binding of PIP2 to the basic region, GTP-CDC42 to the CRIB domain, and several SH3 domain-containing proteins to the poly-proline region, autoinhibition is released. **C.** WASp activation is further stabilized by tyrosine 291 phosphorylation, and the VCA domain can bind the ARP2/3 complex leading to actin polymerization. Modified from Ochs et al (3).

Besides being key to actin cytoskeleton remodeling, WASp can also control gene transcription. Indeed, WASp N-terminal EVH-1 domain controls NFAT-mediated transcription in a way unrelated to actin polymerization, as demonstrated by studies performed on T cell lines transfected with different domains of WASp (75). Studies conducted on more relevant cell types gave further support to this hypothesis. Indeed, $CD4^+$ and $CD8^+$ untransformed T cell lines derived from WAS patients showed a delayed kinetic of NFAT-1 nuclear translocation, and a defective transcription of T-bet and Th1 cytokine genes, following TCR stimulation (27). Similarly, in primary T cells isolated from *was*$^{-/-}$ mice, a delayed kinetic of NFAT-1 nuclear translocation after TCR engagement was described. In addition, a defective nuclear localization of p-Erk, the consequent lack of Elk-1 phosphorylation, and c-fos transcription, led to impaired AP-1 DNA-binding activity. These defects were likely causative of impaired IL-2 gene transcription (76). In line with this hypothesis, it has recently been reported that WASp deficiency in NK cells delays nuclear translocation of NFAT-2 and RelA, a subunit of NFκB, in a way independent from actin polymerization (77).
In conclusion, WASp is a molecule whose stability and function is tightly regulated, and which is able to integrate a wide array of different inputs to activate actin polymerization, and gene transcription.

2.3.3. *WAS* gene mutations and genotype/phenotype correlation

Approximately 300 unique mutations have been reported in the *WAS* gene, spanning all 12 exons (http://homepage.mac.com/kohsukeimai/WASp/WASPbase.html). It has been demonstrated that the genotype of *WAS* mutations largely determines WASp expression levels (78), which in turn determines disease severity (4, 79). Indeed, missense mutations usually resulting in residual expression of a mutated protein are associated with XLT (disease score 0.5-2, see Figure IV). In contrast, nonsense mutations, deletions or insertions abolishing WASp expression are associated with a full-blown WAS phenotype (disease score 3-5, see Figure IV). However, about 20% of the WASp-expressing patients have a severe clinical phenotype (Figure IV). Moreover, it has been reported that WASp-expressing patients can progress to a score of 5 due to autoimmunity (4) or malignancy (45). Therefore, long-term prognosis cannot be precisely predicted on the basis of mutation and protein expression analysis.

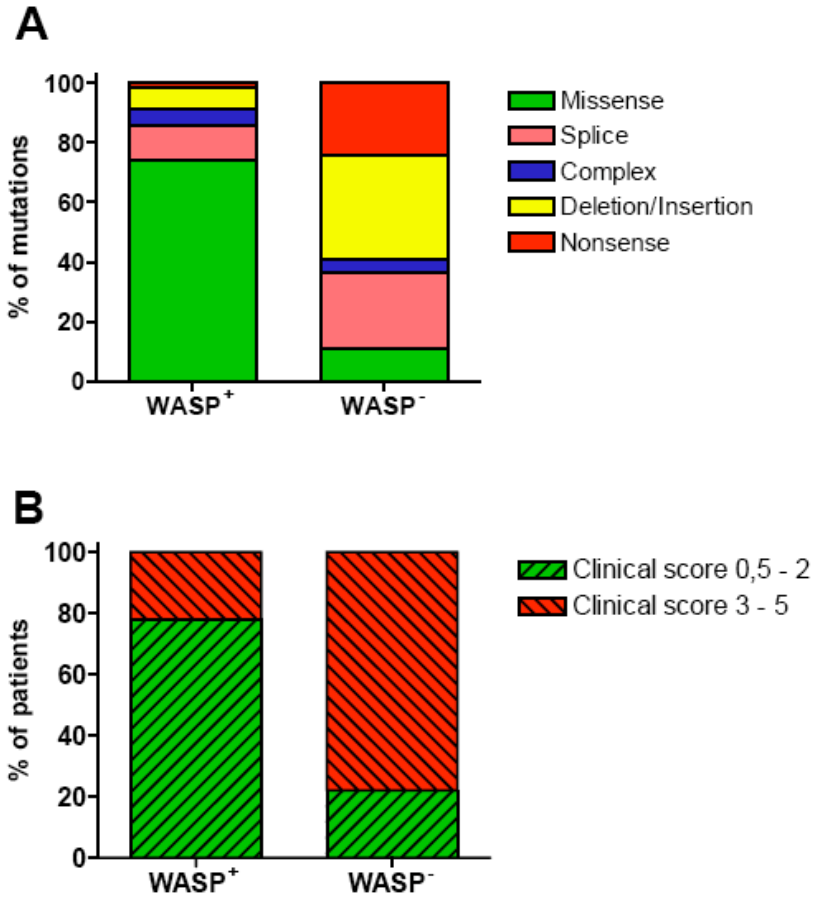


Figure IV: Genotype/phenotype correlation.
A. Correlation between mutation type and WASp expression. Data are derived from Jin et al (79). **B.** Correlation between WASp expression and clinical score. Data are derived from Notarangelo et al (80).

2.4. CELLULAR FUNCTIONS REGULATED BY WASp

2.4.1. 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 kept along hematopoietic differentiation (81). Despite this, the cytological appearance of bone marrow of WAS patients is often normal (14), and $CD34^+$ cells isolated from a WAS patient can differentiate into a normal number of myeloid colonies *in vitro* (82). Therefore, WASp might be dispensable in early haematopoiesis.

The study of obligate female carriers of a mutated *WAS* allele shed light on the function of WASp in hematopoietic stem cells. 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 can play a role in the lyonization process (83). However, a direct role for WASp in X-chromosome methylation has never been demonstrated. Alternatively, non-random X-inactivation in the bone marrow can be explained by a migratory defect of WASp-null HSC. Analysis of heterozygous $was^{+/-}$ mice, and competitive transplantation experiments, demonstrated that WASp is crucial for transition of hematopoietic stem cells from the fetal liver to the bone marrow, and for their engraftment (37). Besides providing a possible explanation to the non-random X-inactivation, these findings are highly relevant for WAS gene therapy, as they predict the preferential engraftment of gene corrected hematopoietic stem cells.

2.4.2. T lymphocytes

T cell defects, affecting effector, helper and regulatory functions, are thought to play a key role in WAS-associated immunodeficiency.

WASp plays a key role in T cell activation and actin cytoskeleton remodeling after the engagement of the T cell receptor (TCR) (29, 49, 50, 84), and the co-stimulatory molecules CD28 (66) and CD2 (85). T cell activation is regulated by the formation of the immunological synapse (IS), a polarized cluster of the TCR, co-stimulatory

molecules, signaling molecules, and integrins to 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 a concentric 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 and the IS through Nck (86), which can directly interact with WASp, and/or by the adaptor protein CrkL, which binds WIP and recruits the WIP-WASp complex (58). In addition, WASp recruitment to the IS could be mediated by CD2 through the adaptor molecules CD2AP and PSTPIP1(85). TCR ligation also causes PKCθ-dependent phosphorylation of WIP and disengagement of WASp from the WIP-WASp complex (58). In parallel the Tec tyrosine kinase Itk mediates the TCR-induced recruitment of Vav to the IS (87, 88), which in turn activates CDC42. GTP-CDC42 finally activates WASp *in situ* (86, 89). Alternatively, as mentioned above, Fyn-mediated tyrosine phosphorylation might activate WASp independently of binding to GTP-bound CDC42 (69, 71). For a schematic representation of the mechanisms leading to the recruitment of WASp to the IS, refer to Figure V. In the absence of WASp, IS can be formed only after strong TCR stimulation (90). In addition, lipid raft dynamics during IS formation (28), and IS stability (91) are compromised. Another level of regulation of T cell activation is achieved by prompt internalization of the TCR and the CD28 co-stimulatory molecule after specific engagement, functions that are defective in WASp deficient cells (65, 66). As a consequence of impaired signaling through the TCR and co-stimulatory molecules, T cells from WAS patients and *was*$^{-/-}$ mice show defective proliferation as well as impaired production of IL-2 (27, 29, 49, 50). The inability of *was*$^{-/-}$ cells to proliferate upon initial stimulation precludes the ability in the following downstream events such as Th differentiation and the production of effector cytokines. Indeed human WASp deficient T cells display an impaired production of IFN-γ, while the murine counterpart can produce Th1 cytokines but are defective in their secretion (27, 29, 49, 50, 92). These defects are associated with delayed NFAT-1 nuclear translocation and defective T-bet induction (27, 76). Very

recently WASp was also shown, in mice but not in humans, to control the Th2 effector function of CD4 T cells. Morales-Tirado et al. in fact demonstrated a post-transcriptional requirement for WASp in IL-4 production (93).

Notably even natural occurring regulatory T cells (nTreg), a key population in the control of autoimmunity, are dysfunctional in the absence of WASp (42-44). Indeed, similar as for the other T cell subsets, WASp is required for the TCR/CD28-triggered proliferation, for TGF-β production and for the suppressive function of nTreg.

In addition to its role in T cell activation and cytokine production, WASp is also critical for T cell chemotaxis *in vitro* in response to SDF-1α (94) and *in vivo* homing to secondary lymphoid organs (95).

A reduction in the numbers of circulating naïve $CD4^+$ and $CD8^+$ T cells may be present in WAS patients, especially at young age, contributing to the immunodeficiency (39). A decreased thymic output has been hypothesized based on the description of abnormal thymic involution in a limited number of histological studies (96, 97). On the other hand, recent studies have highlighted that WASp is dispensable for thymic generation of T cells in mice. Indeed, *was*$^{-/-}$ mice have a relatively normal thymic development, but abrogation of both WASp and N-WASp function through a dominant negative portion of WASp (98), or simultaneous knock-out of *N-Was* (53), cause 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 Vβ repertoire is normal in young WAS patients, indicates that WASP absence does not impair thymopoiesis qualitatively (99). In the same study, it has been observed that the TCR Vβ repertoire of WAS patients becomes skewed after 15 years of age (99). This finding supports the hypothesis of defective T cell survival in the periphery. Accordingly, T lymphocytes isolated from the blood of WAS patients are abnormally prone to spontaneous *in vitro* apoptosis due to decreased Bcl-2 (100), or increased Fas levels (101).

Despite the above information, the precise relationship between T cell abnormalities and WAS-associated immune deficiency, autoimmunity and cancer remains to be elucidated.

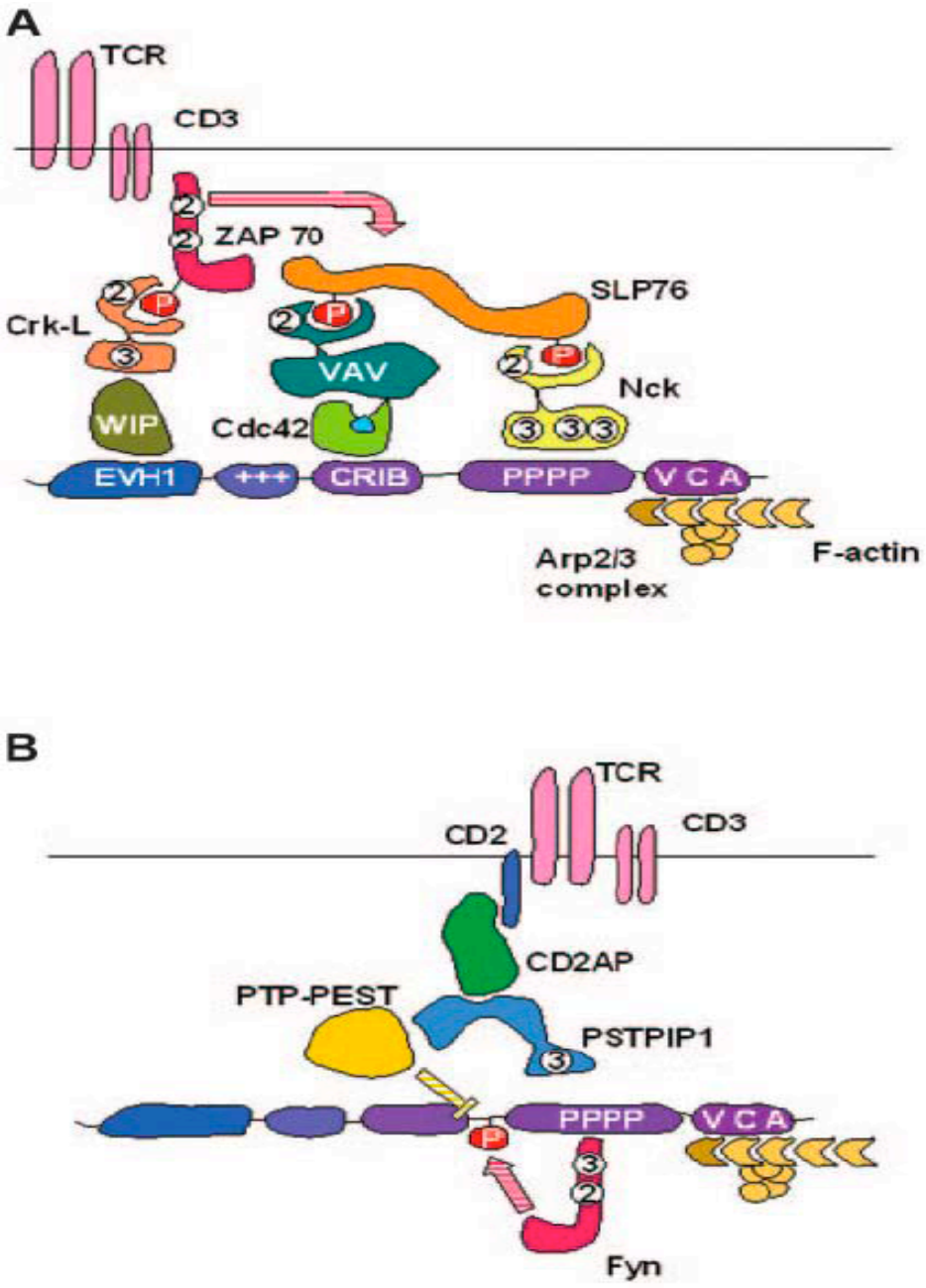


Figure V: Mechanisms of WASp recruitment to the IS in T lymphocytes.
A. Recruitment through WIP and Nck (58, 86, 89). **B.** Recruitment through PSTPIP1, CD2AP and CD26 (69, 85). SH2 and SH3 domains are indicated by "2" and "3", respectively. P indicates phosphorylation. Modified from Burns et al (102).

2.4.3. B Lymphocytes

WAS is also characterized by impaired humoral immunity. Indeed, WAS patients often have elevated IgA and IgE titers. Serum IgM levels are decreased in some patients, but in other ones they are elevated and predict a poor prognosis (24). In addition, WAS patients present a specific defect in the production of Abs against polysaccharide antigens (14).

In contrast to what observed in T lymphocytes, activation of WASp-deficient B lymphocytes can occur normally. Indeed, measurements of calcium fluxes after BCR engagement highlighted a mild defect in WASp-deficient EBV-transformed B cell lines (103), but not in primary B cells isolated from WAS patients (104). Moreover, B cells isolated from *was*$^{-/-}$ mice did not show any activation defect (49, 50). In addition, *in vitro* immunoglobulin class-switching (32, 105) and presentation of soluble antigens (35) do not appear to depend on WASp.

On the other hand, WASp-deficient B cells show alterations of the cytoskeleton (32, 106) likely due to defective F-actin nucleation (107), impaired cell polarization (32, 106), decreased chemotaxis *in vitro* (32), and decreased homing to B cell areas in the spleen *in vivo* (32). In addition, an abnormal splenic architecture characterized by involution of T and B cell areas, and marginal zone, has been described in both *was*$^{-/-}$ mice (32) and WAS patients (108). Germinal centre reaction can also be impaired (32). This finding is in agreement with the observation that WAS patients may have an increased proportion of circulating germinal centre precursors, and a decreased percentage of CD27^{+} post-germinal centre B cells (105).

WAS patients may also suffer from B cell lymphopenia (105). The underlying causes are unknown, but increased spontaneous apoptosis of WASp-deficient B lymphocytes might cause peripheral destruction (100). In contrast with this hypothesis, two recent studies performed in *was*$^{-/-}$ mice clearly indicate a role for WASp in peripheral homeostasis of mature B cell subsets rather than in apoptosis (109). Alternatively, B cell ontogenesis might be affected by the absence of WASp.

In conclusion, B cell intrinsic defects may act in concert with defective T cell help to generate the impaired humoral immunity observed in WAS patients.

2.4.4. Natural Killer lymphocytes

The high susceptibility of WAS patients to viral infections and lympho-reticular malignancies suggests an impairment of NK cell-mediated cytotoxicity and immune surveillance.

Even though WAS patients have a normal or increased percentage of circulating NK cells (30, 31), these cells have a reduced ability to form conjugates, and fail to polarize actin towards the target cells *in vitro*. Consequently, both direct and CD16-mediated cytolysis (a model of ADCC) are impaired (30, 31). In addition, secretion of GM-CSF is defective in WASp-deficient NK cells (77).

WASp is physiologically recruited to the contact area between NK cells and their targets (the lytic synapse) (31), and is activated by GTP-CDC42 and by tyrosine phosphorylation after CD16 or β2-integrin engagement (30). WASp deficiency in NK cells alters significantly the molecular composition of the lytic synapse, and delays the nuclear translocation of NFκB and NFAT-2 (77).

In addition, defects in other cell types can contribute to the dysfunction of NK cells in WAS patients. To this regard, it has been demonstrated that *in vitro* lytic activity of NK cells isolated from WAS patients can be rescued by treatment with exogenous IL-2 (30), leading to the hypothesis that impaired IL-2 production by WASp-deficient T cells might play a role in overall NK cell dysfunction in WAS. Moreover, it has been shown that *was*$^{-/-}$ mature DCs are unable to activate NK cells *in vitro* (110). Therefore, WASp deficiency triggers mechanisms intrinsic or extrinsic to NK cells, which can cooperate in causing the profound impairment of NK lymphocyte function observed in WAS patients.

2.4.5. Myeloid cells

Defects in myeloid cell functions, such as pathogen clearance and antigen presentation, can contribute to the immunodeficiency in WAS patients.

WASp absence impairs F-actin reorganization and polarization of monocytes (111), macrophages (112), and DCs (113), leading to defects in directional chemotaxis *in vitro*. In macrophages and DCs, migration depends on the formation of podosomes, peculiar cell adhesion structures characterized by an actin core surrounded by an integrin ring. WASp localizes to podosomes through WIP (54), and is absolutely

required for their formation (114).

WASp is also key for efficient phagocytic cup formation in human macrophages, and its deficiency impairs the uptake of opsonised bacteria and apoptotic cells (33, 34, 115).

Impaired DC function in the absence of WASp can result in defective priming of T cells. Indeed, studies on *was*$^{-/-}$ mice have shown that *was*$^{-/-}$ DCs home inefficiently to secondary lymphoid organs (116) and cause defective *in vivo* priming of CD4 and CD8 T cells (117, 118). Moreover, *was*$^{-/-}$ DCs are impaired in presenting particulate antigens to T cells *in vitro* (35). In addition to the above findings, *in vitro* activation of NK lymphocytes by *was*$^{-/-}$ DCs is inefficient (110).

WASp is also key for neutrophil function, as testified by defective cell adhesion, migration, and oxidative burst in WASp-deficient neutrophils activated through the β1, β2, and β3 integrins (36). In a separate study, defective phagocytosis of yeast zymosan particles by *was*$^{-/-}$ granulocytes has been observed (49). WASp is also required for mast cell degranulation and cytokine production after FcεRI engagement (119).

In conclusion, the defects described above are likely to impair pathogen recognition and clearance, which in turn may contribute to WAS-associated immunodeficiency, chronic inflammation, and development of autoimmunity.

2.4.6. Megakaryocytes and platelets

Platelet defects are observed in all WAS patients irrespectively of the severity of the mutation, likely as a consequence of specific protein instability and degradation (13). Whether thrombopoiesis is defective in WAS patients is still a matter of debate. Indeed, the number of megakaryocytes in the bone marrow of WAS patients is generally normal (4, 14), and defective proplatelet production by megakaryocytes derived from WAS patients was reported in one study(15), but excluded by another one (16). To be efficiently released into the blood stream, platelets must be generated in close proximity to, or even within, the blood vessels in the bone marrow. In contrast, a recent study reported that platelets are ectopically released and accumulate within the bone marrow stroma of *was*$^{-/-}$ mice, because *was*$^{-/-}$ megakaryocytes lack negative regulation of proplatelet formation by the collagen receptor α2β1 integrin

(17). This aspect might contribute to defective platelet release in the circulation, but awaits confirmation on human samples.

Peripheral destruction of platelets by the reticulo-endothelial system is thought to play a major role in the pathogenesis of the thrombocytopenia. Indeed, the half-life of WASp-negative platelets is decreased (14, 18, 19, 42), the frequency of platelets co-localizing with macrophages in the spleen of WAS patients is abnormally high (20), and splenectomy can ameliorate WAS-related thrombocytopenia (23). However, how WASp regulates platelet lifespan is largely unknown. Since platelet-associated IgG have been found in some but not all patients (4), it is likely that the general cause of thrombocytopenia is not related to autoreactive antibodies-dependent elimination. To this regard, it has been reported that platelets isolated from WAS patients abnormally expose at their surface the phospholipid phosphatidylserine (PS), a signal for engulfment by macrophages. The underlying mechanism has not been investigated yet (20).

Platelet function is also dependent on WASp. Indeed, WAS patients with normal platelet counts after splenectomy are not completely protected from hemorrhages (23). In platelets, WASp is activated by phosphorylation after engagement of the collagen receptor GPVI (120). In addition, WASp-null platelets show impaired activation after thrombin stimulation (22), and decreased adhesion to fibrinogen through the αIIbβIII integrin (25). Nonetheless, defective *in vitro* aggregation of platelets isolated from WAS patients is an inconsistent finding (14, 18, 120, 121).

Therefore, despite intensive research, the cellular mechanisms causing thrombocytopenia and platelet dysfunction in WAS patients are still largely unknown.

2.5 INVARIANT NATURAL KILLER T (iNKT) CELLS

2.5.1 Identification of invariant Natural Killer T (iNKT) cells

Invariant Natural Killer T (iNKT) cells are narrowly defined as a T cell lineage expressing NK lineage receptors, including CD161 in human and its murine homologue NK1.1 in the C57BL/6 background, in addition to semi-invariant CD1d-restricted αβ T cell receptors (TCRs) (122).

Several lines of research indicated NKT cells as a separate lineage of T lymphocytes. The first observation included the identification of a canonical Vα14-Jα18 rearrangement in a set of hybridoma derived from mouse KLH (keyhole limpet hemocyanin)-specific suppressor T cells (123-125), and later in cDNA extracted from lymphoid organs of unimmunized mice (126, 127). In parallel, other independent studies leaded to the identification of a murine $CD4^{-}CD8^{-}$ double negative T cell subset with a Vβ8 usage bias (128, 129) and of a recurrent Vα24Jα18 rearrangement in human DN peripheral blood lymphocytes (130, 131). These observations were pieced together when a subset of CD4 and DN IL-4 producing thymocytes co-expressing NK lineage receptors was independently identified and shown to express a biased set of Vβ8, Vβ7 and Vβ2 TCR β chains (132-135) combined with a canonical Vα14-Jα18 chain in mouse (136), and with the homologous Vα24-Jα18/Vβ11 pair in human (136, 137).

The finding that mouse and human NKT cells were autoreactive to cells expressing CD1d (137-140), a member of the CD1 family of MHC-like molecules, completed the initial characterization of this lineage and raised new question regarding their specificity, development and function.

2.5.2 Distinctive features of iNKT cells

One of the most important features distinguishing iNKT cells from conventional T cells is the TCR composition, which is semi-invariant in mouse and invariant in human (122). More than 80% of these TCR are Vα14-Jα18/Vβ8, Vβ7 and Vβ2 in mouse (or Vα24-Jα18/Vβ11 in human), while the remaining 20% represents a

collection of rare Vα3.2-Jα9/Vβ8, Vα8/Vβ8 and other TCRs (141, 142). Although both the Vα14 and the non-Vα14 iNKT cells exhibit autoreactivity to CD1d expressing cells, particularly thymocytes, their antigen specificity does not overlap. Thus, mVα14 and hVα24 iNKT cells, irrespective of their Vβ-Dβ-Jβ chain usage, recognize a marine sponge-derived α–GalactosylCeramide (αGalCer) (143, 144) and closely relate microbial α-glycuronylceramides (145-147), as well as the self-antigen isoglobotrihexosylceramide (iGb3) (148). In contrast, the self and foreign antigens recognized by non-Vα14 NKT cells remain to be identified. Thereon I will focus on the canonical mVα14 and hVα24 iNKT cells, which I will simply refer as iNKT cells, because they are the best-characterized elements of the NKT cell family. Indeed the generation of αGalCer loaded CD1d tetramers (149), the golden tool for iNKT cell detection, allowed the achievement of new important findings in iNKT cell biology.

Many iNKT cells in human and mice express CD161, a cell surface molecule usually observed on NK cells that correspond to the NK1.1 antigen, and that is the reason why they are referred as Natural Killer T cells. Nevertheless not all αGalCer/CD1d tetramers^{+} T cells express CD161 (150). Murine and human iNKT cells express additional receptor commonly found in NK cells. iNKT cells in C57/BL6 mice express intermediate level of IL-2 receptor β (CD122). Moreover the expression of the inhibitory NK receptor Ly49A and Ly49G2 has been reported on iNKT cells (151), as well as the expression of other NK markers as NKG2D and DX5 (CD49b) (122, 152).

A striking feature of most murine and human iNKT cells is their expression of marker associated with recently activated or memory T cells. In mice, most iNKT cells are CD44^{hi}CD69^{int}CD45R^{hi}BCD62L^{lo}CCR7^{neg}, while in humans CD45RO^{+}CD45RA^{-} CD25^{+}CD62L^{-}CCR7^{-}, and only 5%-15% express CD69 (153-155). Interestingly, iNKT cells in human cord blood and in germ-free mice display this activated/memory surface phenotype, suggesting that previous exposure to foreign microbial antigens is not the reason for this phenotype and that stimulation by CD1d-presented self ligands is likely to be sufficient (154-156).

In addition iNKT cells in mice, and to a lesser extent in humans, express intermediate levels of TCR at the cell surface, which may be the consequence of continuous low-

level TCR stimulation provided by recognition of self antigens that are constitutively presented by CD1d (150).

Based on CD4 expression, two different iNKT cell subset, with different functional properties, surface markers expression and tissue distribution, can be distinguished: the $CD4^+CD8^-$ ($CD4^+$) and the $CD4^-CD8^-$ (double negative, DN) iNKT cells (157). In general, in mice the majority of iNKT cells is $CD4^+$, while in humans a mean of 50% of αGalCer/CD1d $tetramers^+$ T cells is $CD4^+$, with high donor-to-donor variability (150). However a depth analysis of different subsets revealed marked differences in tissue distribution and cytokine production. For instance whereas $CD4^+$ iNKT cells represent the major subset in thymus, spleen and liver, they are a relatively minor subset in lymph nodes and bone marrow (157). In human, but less clearly in mice, this classification provides an important functional distinction, since $CD4^+$ iNKT cell make both Th1 and Th2 cytokines, whereas $CD4^-$ iNKT cells mainly make Th1 cytokines (158).

2.5.3 Tissue Distribution and Localization of iNKT cells

In mice, αGalCer/CD1d $tetramers^+$ T cells are approximately 0.5-1% in thymus, 1-2% in spleen, 0.5% in lymph nodes, 10-50% in liver, 0.5% in bone marrow and 1% of intestinal intraepithelial lymphocytes (IEL) of the total lymphocytes per organ (149, 159). In humans, iNKT cells account for a mean of 0.2% of peripheral blood T cells, as determined by the use of both Vα24Vβ11 antibodies and αGalCer/CD1d tetramers (131, 158). Moreover the number of iNKT cells in human liver is lower than in the murine counterpart.

Many iNKT cells display chemokine receptor and homing molecule profiles which are more similar to those of effector (memory) T cells than naïve T cells. For instance in humans the majority of iNKT cells express CCR5, CXCR3 and CXCR6, chemokine receptors associated with Th1 responses and migration to sites of inflammation. Only a small fraction of human iNKT cells express CCR7 and no circulating iNKT cells express CXCR5, receptors required for migration into T and B cell zones of lymphoid organs (158, 160, 161). In mice most iNKT cells express CXCR3 and migrate robustly to MIG/CXCL9. Although a large proportion of murine iNKT cells express also the chemokine receptor CXCR6, they weakly migrate to the

CXCR6 ligand (CXCL16) (162). Their expression of CXCR6 matches the expression of CXCL16 on the endothelial cells lining the liver sinusoids and appears to be important for survival rather than for migration to the liver (163).

Only murine splenic NK1.1$^-$ iNKT cells, considered immature cells recently released from the thymus (164, 165), migrate in response to the CCR7 ligand SLC/CCL21(162). Interestingly only iNKT cells from the spleen, but not liver, migrate in response to the CXCR5 ligand BCA-1/CXCL13, suggesting that these cells could be recruited to B cell-rich areas in the spleen and thus modulating the follicular B cell responses (162).

Only a small fraction of human and murine iNKT cells expresses high level of L-selectin (CD62L), that would allow entry into secondary lymphoid organs via high endothelial venules (HEVs), suggesting that iNKT cells recirculate through peripheral tissue and enter lymph nodes most likely through the afferent lymphatics rather than HEVs (150).

Moreover CD4$^+$ and DN iNKT cell subsets can present differences in chemokine receptor expression (160, 161) and in chemotactic responses (162), indicating that they may be recruited to different sites.

2.5.4 Ligands of iNKT cells

There is nowadays a general consensus that CD1d, like other CD1 family members, evolved to present lipid to T cells (150). However the nature and the source of the various lipids naturally binding to CD1d remain poorly elucidated.

Kirin Pharmaceuticals identified the first ligand of iNKT cells during a screening for the identification of antitumoral compounds. During this study, they found that extracts from the marine sponge *Agelas mauritianus* prolonged the survival of mice bearing B16 melanoma (166). The active principle was an α-branched galactosylceramide and it was slightly modified to produce the compound commonly known as αGalCer (Figure VI) (167). The lipid nature of this compound was demonstrated to induce a strong CD1d-restricted and TCR-dependent activation of iNKT cells (143).

The αGalCer structure resembles mammalian ceramides since it contains a sphingosine-like base, an amide linked acyl chain and an O-linked pyranose (Figure VI). However the anomeric carbon of the sugar is in the α-linkage to the oxygen, whereas in mammals the corresponding linkage is of the β-anomeric type (150). Although αGalCer has no physiological function in mammalian immunity, it has been broadly used as a tool to study iNKT cell activation. Irrespective of their variable CDR3 β sequence, most of mouse and human iNKT cells recognize αGalCer, and the mouse CD1d-αGalCer tetramers stain mouse, human and non human primate iNKT cells as well, providing a clear evidence of the high degree of conservation of this recognition system (144).

Several independent groups reported that also some microbial glycolipid antigens could directly activate iNKT cells by engaging their invariant TCR. Indeed in 2005 three reports described glycosphingolipid antigens from *Sphingomonas* spp, activating essentially all iNKT cells (figure VI) (145-147). Glycosylceramides from the cell wall of *Sphingomonas* spp display structural features similar to αGalCer, including the unusual α-linkage of the sugar to the sphingosine-containing lipid. They can bind CD1d and specifically activate mouse and human iNKT cells (145-147). Moreover direct recognition by the invariant TCR was confirmed by staining with CD1d tetramers loaded with glycosphingolipid from *Sphingomonas* spp.

Glycosphingolipids expressed by another bacterium, the causative agent of Lyme disease *Borrelia burgdorferi*, can directly stimulate iNKT cells (168). However recognition of intact or heat-killed bacteria could not be demonstrated and only one isolate report has suggested defective bacterial clearance *in vivo* in mice lacking iNKT cells (169).

In addition, various CD1d-expressing cell types can stimulate mouse and human iNKT cells at low-level, in the absence of foreign microbial antigens (137, 139, 170). This autoreactivity, together with the presence of IL-12 induced by TLR engagement, was shown to be required for iNKT cell activation during immune responses against Gram negative, LPS positive bacteria (145, 171). Moreover autoreactivity may explain the thymic development of iNKT cells (140), which includes an expansion phase following the positive selection (164) and the acquisition of a memory phenotype independent of microbial exposure or TLR signaling (156). The endogenous glycosphingolipid iGb3 (Figure VI) induces a weak activation of mouse

and human iNKT cells upon presentation by DC or plastic-bound CD1d/iGb3 preformed complexes (148, 172). iGb3 was hypothesized to be the endogenous ligand essential for the positive selection of mouse iNKT cells in the thymus. Indeed mice deficient in the β-subunit of β-hexosaminidase, an enzyme involved in the metabolism of lysosomal glycosphingolipid including iGb3, exhibit greatly reduced frequency of iNKT cells (148). However a normal iNKT cell frequency was found in mice lacking iGb3 synthase (iGb3S), which had no detectable isoglobo-series (iGb3, -4, and-5) (173), suggesting that the loss of iNKT cells in the previous model probably results from lipid storage alterations per se rather than the specific absence of iGb3. Further studies will be required to unravel the identity of the endogenous ligand/s involved in iNKT cell positive selection and peripheral activation.

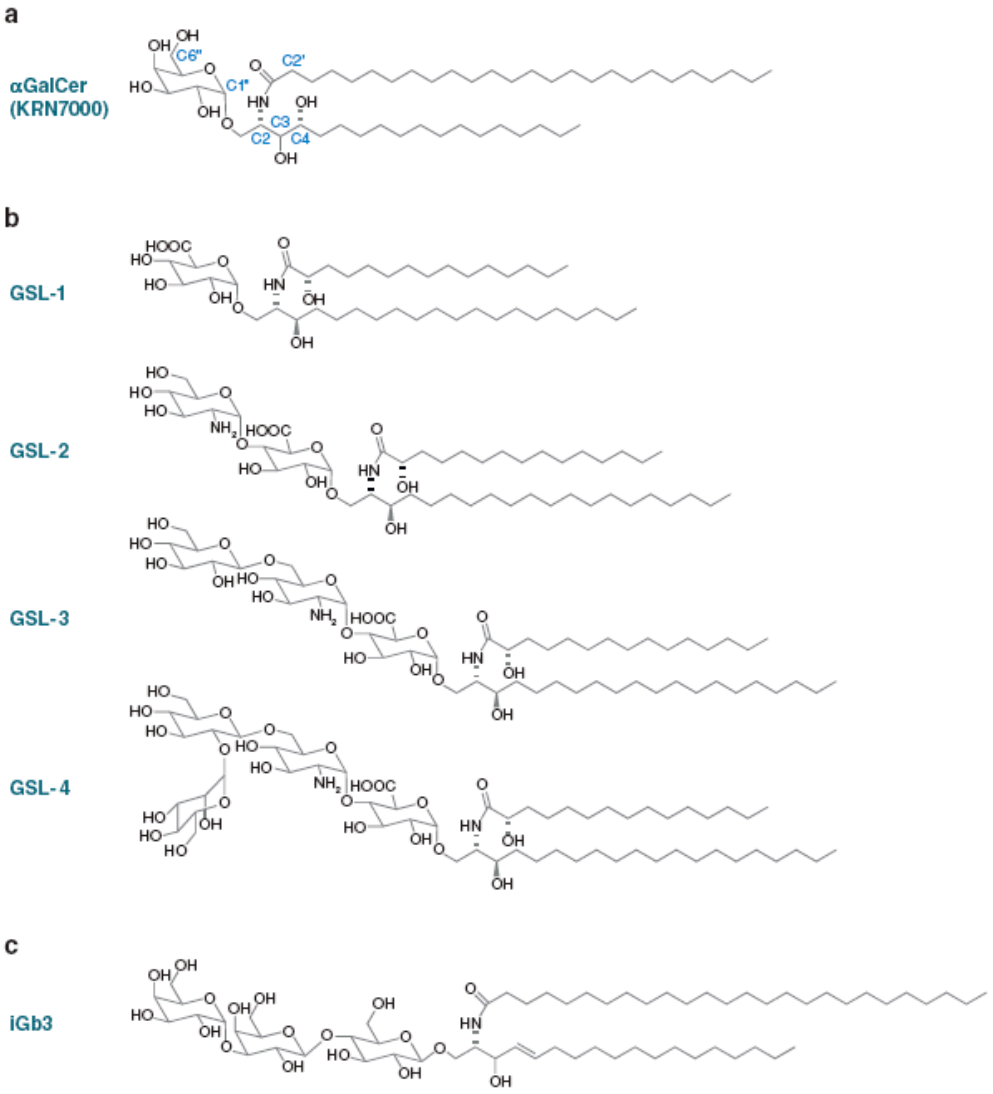


Figure VI: Self and microbial glycosphingolipid ligands (GSL) of NKT cells.
A. Marine sponge αGalCer (KRN7000) with carbon atom number assignments on sphingosine (C), acyl (C'), and carbohydrate (C''). B. *Sphingomonas* GSL-1 through GSL-4. C. Mammalian isoglobotrihexosylceramide (iGb3), or Galα1,3Galβ1,4Glcβ1,1Cer. Note that the proximal glucose of the mammalian glycosphingolipid has a β-anomeric linkage to ceramide, in contrast with the α-branched galactose of αGalCer or glucuronyl of *Sphingomonas* GSLs. Modified from Bendelac et al (122).

2.5.5 Cell biology of lipid presentation by CD1d

CD1d is constitutively expressed by APCs. In fact DCs, macrophages and B cells (in particular marginal zone B cells) express CD1d at high level (174, 175).

Also cortical thymocytes express CD1d, and this expression is mandatory for the development of iNKT cells (140). Moreover CD1d is expressed on hepatocytes, Kupffer and endothelial cells lining liver sinusoids, where iNKT cells are most abundant in mice (163). However in the liver CD1d expression is not required for iNKT cell homing (176). In addition, similar to the MHC class II molecules, most other solid tissue cells and non-antigen-presenting hematopoietic cells express low or undetectable levels of CD1d.

Within the lumen of the endoplasmic reticulum (ER), newly synthesized CD1d molecules interact with the chaperone proteins calnexin, calreticulin and the thiol oxidoreductase ERp57 (177), which are involved in the association of CD1d with β2-microglobulin (β2-m). CD1d molecules can traffic to the cell surface in the absence of β2-m. Mutation analysis revealed that the loss of glycan-2, at the interface between CD1d and β2-m, results in faster CD1d egress from the ER and reduced stability at the cell surface (178). Supporting the hypothesis that lipid ligand binding to CD1d can occur in the ER, it was shown that CD1d molecules engineered to be retained in the ER bound phosphatidylinositol (179).

From the ER, CD1d molecules traffic to the cell surface through the secretory pathway before being reinternalized into the endo-lysosomal compartment. This recycling between the plasma membrane and the endo-lysosomal compartment is dependent on a tyrosine motif encoded in the CD1d cytoplasmic tail (180, 181). The tyrosine motifs allow CD1d to bind to the specific adaptors protein (AP) complexes AP-2 and AP-3, which in turn direct CD1d trafficking. The intracellular localization of human CD1d is different in mice and in humans: although both are found within early and late endosome as a result of binding AP-2, murine CD1d also binds AP-3, explaining why most murine CD1d molecules are found within lysosome (182-184).

Mutations or deletions of the cytoplasmic tail motif and modifications of CD1d trafficking to acidified lysosomes in mice with a defect in AP-3 impair antigen presentation and iNKT cell development (180, 185, 186), indicating the importance of trafficking within endosome and lysosome for the presentation of endogenous ligand

and, consequently, the thymic generation of iNKT cells. See figure VII for an overview of CD1d trafficking.

A number of endosomal proteins are involved in the processing and loading of glycolipids onto CD1d molecules (187). These proteins include saposins, Niemann-Pick type C2 (NPC2) protein, and microsomal triglyceride transfer protein (MTP). Saposins A, B, C, and D are produced from the proteolytic cleavage of prosaposin within the endosome. Prosaposin-deficient mice have defective iNKT cell development and reduced ability to process and present glycolipid antigens (188). Loading of human CD1d with αGalCer in the absence of saposins is similarly impaired (189). Individual saposins differ in their ability to load particular lipid, indeed saposin B is the most efficient of the saposins in transferring glycolipids onto CD1d for presentation. Mice lacking the NPC2 protein, a lysosomal lipid transfer protein, have impaired presentation of glycolipids and impaired selection of iNKT cells in the thymus (190). It was shown that NPC2 dimers can bind iGb3 and facilitate its loading onto CD1d molecules. Finally MTP, another lipid transfer protein expressed in the ER was found to co-precipitate with CD1d and genetic or drug-induced inhibition of MTP was associated with defect in lipid antigen presentation.

Interestingly a recent report from Im J.S. and colleagues demonstrated that not all the glycosphingolipid antigens display the same requirement for endosomal loading (191). Indeed they found that αGalCer analogues, able to induce biased Th2 cell type responses, were presented with rapid kinetics and without a requirement for intracellular loading of CD1d, apparently as a result of their ability to rapidly associate with CD1d molecules directly at the cell surface. This was in marked contrast to classical αGalCer, which underwent intracellular loading and was presented more slowly.

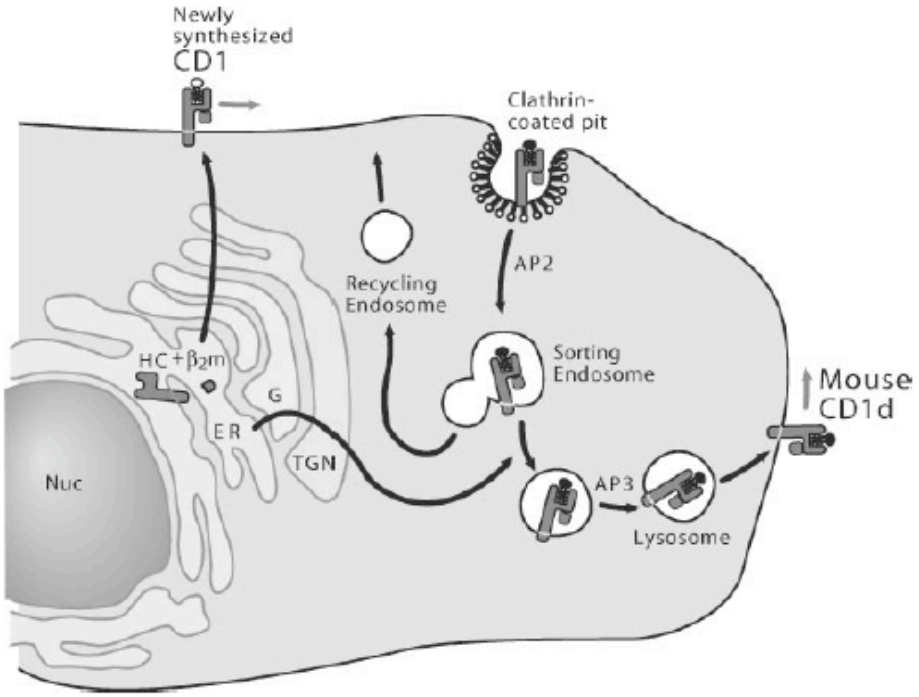


Figure VII: Intracellular trafficking of CD1d.
Newly synthesized CD1d molecules may or may not assemble with β2-microglobulin in the ER, acquire self-lipids (*open circle with zigzag tail*), and traffic to the plasma membrane along the secretory route. CD1d molecules are internalized from the plasma membrane and traffic through the early and late endosomal compartment and are delivered to lysosomes in an AP3-dependent manner. CD1d then acquires distinct self-lipid antigens (*filled circle and zigzag tail*) in late endosomal/lysosomal compartments. Human CD1d traffics mainly in early and late endosomes but not in recycling endosomes and only partially localizes in lysosomes. Moreover human CD1d does not associate with the adaptor protein AP3. Modified from Brigl and Brenner (150).

2.5.6 iNKT cell development

For many years the developmental origin of iNKT cells was a matter of debate (152). Although iNKT cells are present in the thymus, their origin has remained controversial mostly because these cells were first identified by using surrogate markers such as NK1.1, which are also expressed by other cells.

Some studies suggested that iNKT cells develop very early in ontogeny, independently from the thymus, and before the appearance of conventional T cells (192, 193). However, there are now compelling evidences that iNKT cells are a thymus-dependent population. Indeed they are absent in nude mice (165, 194); do not develop in thymectomized mice (195-197) and are detectable in the thymus shortly later than other T-cell subsets (164, 165, 198-200).

Different models that explained the basis of the iNKT cell lineage were initially hypothesized (150). The "committed precursor" model suggests that iNKT cells originate from precursors committed prior to T cell receptor (TCR) expression. On the contrary the "selective" model proposes that the development of iNKT cells is a selective event stemming from the random production of a TCR that recognize CD1d (152). Convincing evidences support the last model. Indeed although iNKT cells always express the invariant TCR Vα14-Jα18 chain (136), their non-transcribed TCRα allele shows clear evidence of random recombination (201). Furthermore, although the complementarity-determining region 3 (CDR3) of the α-chain is invariant at the aminoacid level, this is not the case at the nucleotide level, in which non-templated nucleotide additions contribute to the invariant amino-acid sequence (136).

It is so far commonly accepted that iNKT cell lineage in mice branches from the developmental pathway of conventional T cells at the $CD4^+CD8^+$ DP stage (200, 202, 203) (Figure VIII). This event occurs when DP thymocytes, bearing the randomly generated TCR of iNKT cells, undergo to positive selection (152). The positive selection of iNKT cells requires their ligation to endogenous glycolipid antigens presented by CD1d on DP cortical thymocytes. This was shown using bone marrow chimeras or transgenic restoration of CD1d expression in *CD1d*$^{-/-}$ mice, to restrict the expression of CD1d to the hematopoietic or stromal compartment of mice. iNKT cells were selected only when CD1d was expressed by hematopoietic cells, and more

specifically by DP thymocytes (140, 176, 196). Of note the presence of CD1d alone is not sufficient to sustain iNKT cell selection. In fact several studies have shown that CD1d also needs to be able to recycle through the intracellular endo-lysosomal pathway (122). In the absence of this trafficking CD1d can be expressed on the cell surface, but iNKT cell selection is absent or deeply impaired. This finding strongly suggest that iNKT cell selection requires CD1d to be loaded with an endogenous endosome- or lysosome-derived antigen(s).

An interesting issue about the positive selection of iNKT cells is related to their biased usage of TCR Vβ chains (Vβ8, Vβ7 and Vβ2 in mice, Vβ11 in humans). Indeed it is tempting to speculate that it is caused by the inability of the invariant Vα chain to pair with other Vβ chains. However a recent study on TCR transgenic *CD1d*$^{-/-}$ mice revealed that the invariant Vα14-Jα18 TCR could paired with many different Vβ chains (204). By contrast the Vβ8.2, Vβ7 and Vβ2 chains were required for the recognition of iGb3, suggesting that the selecting glycolipid antigen determines the TCRβ usage. Even among the selected Vβ chains some appear to confer higher affinity for antigen presented by CD1d. Indeed Vβ8.2 is expressed by the majority of iNKT cells and provides the highest affinity for αGalCer-CD1d recognition (205) whereas Vβ7^{+} iNKT cells are preferentially selected under competitive conditions (in *CD1d*$^{+/-}$ mice) and preferentially expanded by DP thymocytes unpulsed (presumably presenting an undefined self-antigen) or pulsed with iGb3 (206).

Some evidences suggested that iNKT cells, as conventional T cells, might be susceptible to negative selection. Supporting this hypothesis αGalCer administration during iNKT cell development abrogated *in vitro* or *in vivo* iNKT cell development (207, 208). In addition, even in the absence of exogenously added ligands, over-expression of CD1d on DCs prevents iNKT cell development (207). These data indicate that the avidity for a self-ligand presented in the context of higher levels of CD1d hampers iNKT cell development. Nevertheless, up to now, there is no direct demonstration of the deletion of iNKT cell precursors and no CD1d tetramer positive iNKT cell subset has been directly shown to be susceptible to negative selection.

The developmental stages of mouse iNKT cells can be followed based on CD24, CD44 and NK1.1 expressions (122, 152) (Figure VIII). The first detectable stage of iNKT cell development (Stage 0) has a CD24^{high} phenotype and includes a

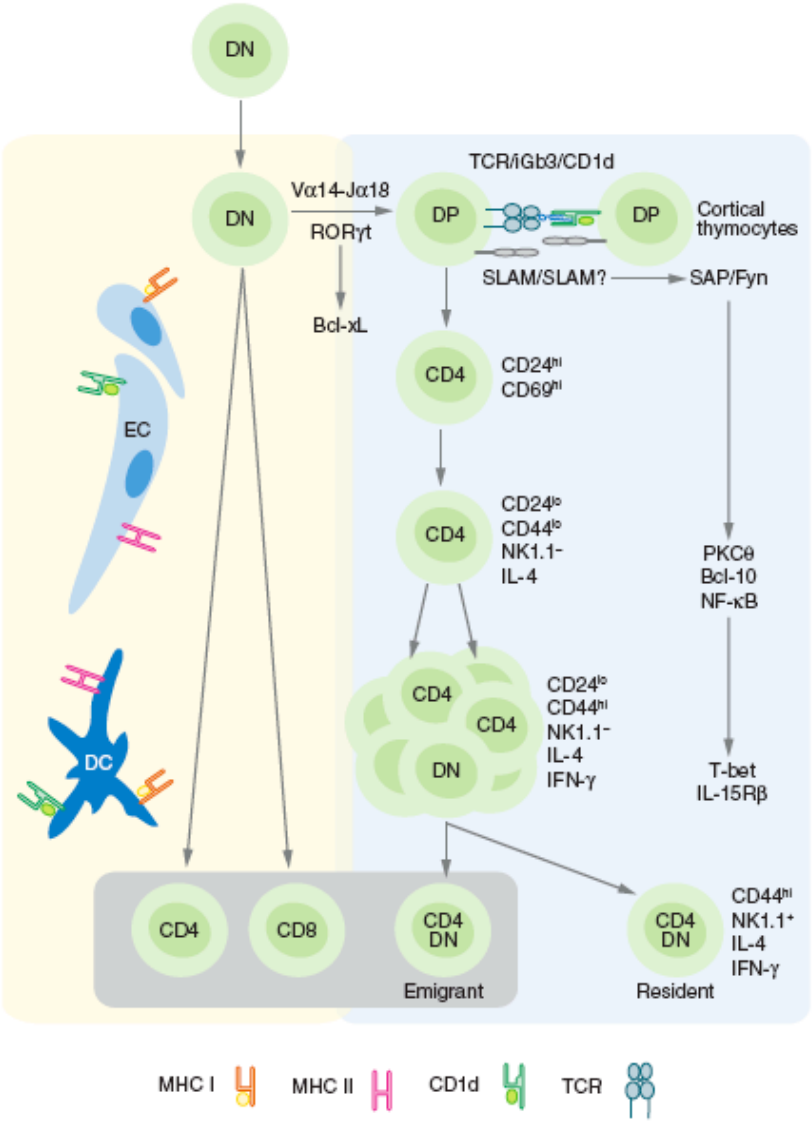


Figure VIII: Developmental pathway of murine iNKT cells.
NKT cell precursors diverge from mainstream thymocyte development at the CD4+CD8+ double-positive (DP) stage. Upon expression of their canonical TCRα chain, which requires survival signals induced by RORγt, NKT cell precursors interact with endogenous agonist ligands such as iGb3, presented by CD1d expressed on other DP thymocytes in the cortex. Accessory signals provided through homotypic interactions between SLAM family members recruit SAP and Fyn to activate the NF-κB cascade. DP precursors downregulate CD8 to produce CD4+ cells, and a subset later downregulates CD4 to produce CD4−CD8− double-negative (DN) cells. Unlike mainstream T cells, NKT cell precursors undergo several rounds of cell division and acquire a memory/effector phenotype prior to thymic emigration. Acquisition of NK lineage receptors, including NK1.1, occurs after emigration to peripheral tissues, except for a minor subset of thymic NKT cell residents. The transcription factor T-bet is required for induction of the IL-15 receptor β chain and survival at the late-memory and NK1.1 stages. EC, epithelial cell; DC, dendritic cell. Modified from Bendelac (122).

$CD4^{intermediate}CD8^{intermediate}$ DP stage, followed by a $CD4^{high}CD8^{neg}$ stage. These developmental intermediates immediately follow positive selection, as they express CD69 and are not found in the thymus of *CD1d*$^{-/-}$ mice. However at Stage 0 iNKT cells are present at very low frequency (10^{-6}**)**, they are small and apparently non dividing, suggesting that the extensive post-selection expansion of iNKT cells occurs later in the development (198). As cells progress in the maturation, they down-regulate CD24 becoming $CD24^{low}$. Three more developmental stages of $CD24^{low}$ iNKT cells have been described: a $CD44^{low}NK1.1^{neg}$ stage (Stage 1), followed by a $CD44^{high}NK1.1^{neg}$ stage (Stage 2) and finally by the mature stage $CD44^{high}NK1.1^{pos}$ (Stage 3) (122, 164, 165). This sequence is characterized by a massive cellular expansion between Stage 1 and Stage 2 (122). Furthermore during these stages a DN population arises by down regulation of CD4, as shown in cell transfer experiments (198) and by genetic fate mapping in ROSA26R reporter mice crossed to CD4-cre deleter mice (202). However little is known about the precise timing of this branch point because DN iNKT cells can be found within both immature ($NK1.1^{-}$) and mature ($NK1.1^{+}$) iNKT cell subsets (165, 198), although in mice most DN iNKT cells are immature.

Most of $CD44^{high}NK1.1^{neg}$ iNKT cells leave the thymus and migrate to peripheral tissue, where they stop proliferating and rapidly express NK1.1 and other NK lineage receptors (164, 165, 209). Indeed it was unexpectedly found that the most recent thymic emigrant iNKT cells do not express NK1.1 (164, 165), which implies that the final developmental step can take place in the thymus or in periphery. Moreover, confirming the precursor status of $NK1.1^{-}$ cells, adoptively transferred $NK1.1^{-}$ iNKT cells could progress to $NK1.1^{+}$ stage but not vice versa (164, 165, 199). Interestingly, some $CD44^{high}NK1.1^{neg}$ iNKT cells do not migrate to the periphery but undergo to further maturation inside the thymus, where they become long-term resident cells (210). It still remains unclear why the majority of immature iNKT cells are exported to the periphery, since a fraction of iNKT cells can complete the maturation in the thymus. A possible explanation of this phenomenon derives from a recent study, which suggests that peripheral $NK1.1^{-}$ cells could be a peculiar mature iNKT cell subset, rather than a developmental phase. In fact, using adult thymectomized mice, it has been shown that the size of peripheral $NK1.1^{-}$ cells is maintained independently from thymic export (211).

The iNKT cell developmental stages are associated with sharply defined functional changes. Thus $CD44^{low}NK1.1^{neg}$ iNKT cells display a Th2 cytokine profile, since they produce exclusively IL-4 upon *in vitro* TCR triggering, whereas the $CD44^{high}NK1.1^{neg}$ iNKT cells produce both IL-4 and IFN-γ and $NK1.1^{+}$ mature iNKT cells produce more IFN-γ than IL-4 (164, 165).

In humans the study of iNKT cell development was much more difficult than in mice, due to the lower frequency of human iNKT cells. Despite the paucity of information available on human iNKT cell development, it has been identified some interesting parallels with mice. In humans, as in mice, iNKT cells are a population of thymic origin, which migrate from the thymus to the periphery at the $CD161^{low}$ stage (212, 213). However, in contrast to mice, only a minority of thymic iNKT cells expresses CD161, suggesting that in human the final step of maturation occurs in the periphery.

2.5.7 Intracellular signaling pathway regulating iNKT cell development

Over the past years, compelling evidences confirmed the hypothesis that the intracellular signaling requirements for iNKT cell development are different from those of mainstream T cells. This was expected because these two different T cell types are developmentally and functionally distinct.

As discussed above, the interaction with CD1d is mandatory for iNKT cell positive selection in the thymus. However when CD1d is expressed exclusively by DP thymocytes, iNKT cell can pass through positive selection and migrate to the periphery in almost normal numbers, but less of these cells can upregulate the NK1.1 maturation marker (176). In addition adoptively transferred $NK1.1^{-}$ iNKT cell were not able to complete their maturation when transferred in a *CD1d*$^{-/-}$ recipient (209). Together these findings indicate that, beside its role in positive selection, CD1d is also required for the final step of iNKT cell differentiation, which suggests that ongoing TCR ligation with CD1d is part of this process.

Many players regulating iNKT cell development are molecules involved in the TCR signaling. iNKT cell are reduced in numbers and present a more immature phenotype when they lack PKCθ, a signal transduction molecule playing an important role in the TCR induced NF-κB pathway (214). Thus, unlike conventional T cells, which do not require PKCθ for their development and maturation, the ontogeny of iNKT cells

requires PKCθ.

Although the members of the nuclear factor-κB (NF-κB) family have been demonstrated to have a key role in thymic iNKT cell development (214-216), they seem to be dispensable for the ontogeny of conventional T cells. For instance mice expressing an IκBα (inhibitor of NF-κBα) dominant-negative transgene have almost no iNKT cells, revealing a fundamental defect at a very early stage (215). However NF-κB1 deficient mice have a less severe defect in the mature $NK1.1^+$ iNKT cell compartment (215). Defects in alternative NF-κB pathways inhibit the development of iNKT cells with different extents according to which components are targeted. NF-κB2 deficiency causes a two-three-fold reduction in iNKT cell pool (217). However, differently from NF-κB1, which is required in hematopoietic cells, NF-κB2 is required in radiation-resistant non-hematopoietic cells for a proper iNKT cell development (217). The NF-κB family member RelB is necessary in iNKT cell development, as demonstrated by the severe reduction (20 fold) of iNKT cells in RelB deficient mice (217) corresponding with a block in the earliest developmental stages. As for NF-κB2, RelB is required in radiation-resistant non-hematopoietic cells for a proper iNKT cell development (217). In addition a recent study provided evidences that also the NF-κB family member RelA, but not c-Rel, contributes to the regulation of iNKT cell development (218).

Another TCR-induced signaling pathway involved in the regulation of earliest phases of iNKT cell development is that of calcineurin-NFAT-Egr2 (219). Anyway, differently from NF-κB, this signaling pathway is not selectively required by iNKT cells because the disruption of calcineurin B1 and NFAT target *Egr* gene affects also the development of mainstream T cells.

Moreover the over-expression of BATF, an inhibitor of the TCR-induced transcriptional factor AP1, causes a decreased progression from developmental stage 2 to stage 3, whereas it does not significantly alter conventional T cell development (220).

A severe block in terminal iNKT cell maturation was observed in mice lacking T-bet, a transcriptional factor selectively expressed by Th1 but not Th2 cells via a combination of signaling from the TCR and the IFN-γ/STAT1 pathway (221, 222). When iNKT cells lack T-bet, they can upregulate CD44 but fail to express NK1.1 and

complete the maturation program (221). On the other hand normal iNKT cells upregulate T-bet during the progression from stage 2 to stage 3 of development (221). T-bet is strictly required by stage 2 iNKT cells to upregulate IL-15R, which in turn is necessary for IL-15 induced proliferation (221, 222).

The Tec tyrosine kinase Itk, which is important in conventional T cells for the TCR induced Calcium flux generation and NFAT and AP1 activation, was also shown to regulate the generation of a normal pool of iNKT cells as well as the their final maturation steps (223).

Furthermore, a crucial role in the regulation of mature $NK1.1^+$ iNKT cell expansion is played by the costimulatory signal arising from B7-CD28 interaction (224, 225) and by the ICOS/ICOSL interaction (226).

Besides requirement for TCR-induced signals, iNKT cell development is totally dependent on the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) (227, 228). Indeed iNKT cell development is completely abrogated in SAP deficient mice and strongly impaired in individuals suffering from X-linked lymphoproliferative syndrome (XLP), caused by mutation in SAP (227, 228). SAP signals downstream SLAM family of receptor and homotypic interaction between SLAM family members on iNKT cell precursors and DP thymocytes were shown to be required for the transition between positive selection and the subsequent expansion and differentiation of the NKT cell lineage (229). When SLAM molecule are engaged at the cell surface, SAP recruits Fyn, thus initiating signaling cascades leading to NF-kB activation and inhibition of MAPK pathway (152). Of note also Fyn, which is a potent intermediate in the TCR-NF-κB pathway, is required for the development of iNKT cells (230, 231).

Finally a proper iNKT cell development needs the IL-15 induced signaling (232). iNKT cells upregulate the expression of the IL-2/IL-15 receptor β chain (CD122) as they progress from the $NK1.1^-$ to the $NK1.1^+$ stage in the thymus. However IL-15, but not IL-2, is involved in this late developmental stage. In addition IL-15 is crucial for normal iNKT cell turnover and homeostasis (232).

The most representative signaling pathways, which control iNKT cell development, are depicted in Figure IX.

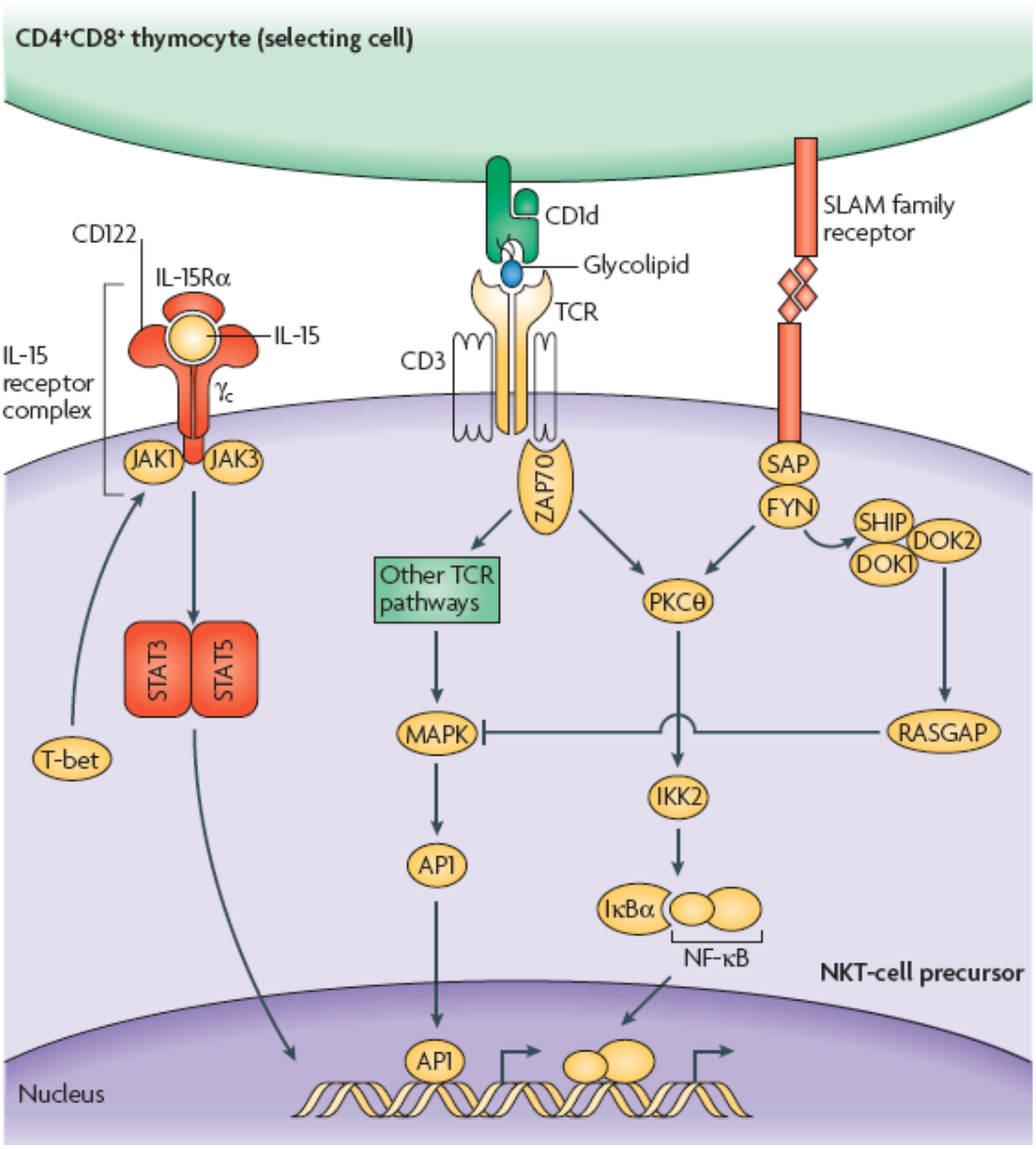


Figure IX: Intracellular signaling pathways that regulate iNKT cell development.
iNKT cell selection and maturation requires a range of signaling events that are not essential for conventional T-cell development, in addition to factors that are common to both T-cell and NKT-cell development. The unique signaling requirements of iNKT cells are not fully understood, but the three main intrinsic pathways that link most of the known mutants that affect NKT-cell development are depicted. These include the SLAM–SAP–FYN pathway, the T-cell receptor (TCR)-signaling cascade (particularly the classical nuclear factor-'B (NF-'B) pathway) and the interleukin-15 (IL-15) pathway.
AP1, activating protein 1; γ_c, common cytokine-receptor γ-chain; DOK, docking protein; IκBα, inhibitor of NF-κBα; IKK2, IκB kinase 2; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PKCθ, protein kinase Cθ; RASGAP, RAS GTPase-activating protein; SAP, SLAM-associated protein; SHIP, SRC-homology-2-domain-containing inositol-5- phosphatase; SLAM, signaling lymphocytic activation molecule; STAT, signal transducer and activator of transcription; ZAP70, ζ-chain associated protein kinase of 70kDa. Modified from Godfrey and Berzins (152).

2.5.8 Activation and effector functions of iNKT cells

Most of the information on the mechanisms leading to iNKT cell activation derives from studies based on the usage of the agonist αGalCer. The events required for αGalCer-induced activation involve TCR ligation by the αGalCer/CD1d complex on APC (e.g. DCs) and interaction of costimulatory molecules, resulting in cytokine secretion and increased CD40L expression on iNKT cells (233). In mice, iNKT cells constitutively express CD28, and its interactions with B7.1 and B7.2 increase IL-4 and IFN-γ secretion upon TCR triggering (234, 235). Through CD40/CD40L interaction and IFN-γ stimulation, DCs are then activated (233, 236, 237). During this cross talk, activated DCs upregulate CD40, B7.1 and B7.2 and produce IL-12, enhancing iNKT cell activation and cytokine production (233, 237). Indeed iNKT cells constitutively express IL-12R, and its expression is upregulated in an IL-12 and IFN-γ dependent manner (233, 238). Although IL-12Rβ2 expression on iNKT cells and CD40 expression on APC are not required for the immediate IL-4 and IFN-γ production by iNKT cells, following stimulation, IL-12 produced by DCs can in turn amplify iNKT cell activation and increase their IFN-γ secretion (233, 235, 237, 239).

However, since αGalCer is neither a microbial nor a self natural antigen of iNKT cells, other mechanisms of iNKT cell activation were unraveled in the last years. As in the case of αGalCer, some microbial glycosphingolipid antigen (e.g. glycosphingolipid from *Sphingomonas* spp. and galactosyl-diacylglicerols from *Borrellia burgdorferi*) can be presented by CD1d and directly activate iNKT cells by engaging their TCR (240) (Figure X). TLR-mediated DC activation, inflammatory cytokines as IL-12 or recognition of endogenous antigen are not involved in this response (240). However it has been described also the existence of indirect activation mechanisms, which do not depend on iNKT cell recognition of microbial antigens. For instance, as demonstrated for *Salmonella typhimurium*, iNKT cells can be activated by the combination of IL-12 produced by LPS stimulated DCs and recognition of endogenous antigen presented by CD1d (171, 240). In addition, another mechanism of indirect iNKT cell activation was shown in a study of the response to *Schistosoma mansoni* (240, 241). Indeed *Schistosoma mansoni* egg sensitized-DCs induce IFN-γ and IL-4 production by iNKT cells. In this response, TLR-mediated activation of DCs is not involved, whereas recognition of endogenous antigen is

required. Finally, it recently emerged a third indirect activation mechanism based on IL-12 and IL-18 production by LPS-activated DCs (242). This mechanism does not require endogenous antigen presentation by CD1d. iNKT cell activation mechanism are represented in Figure X.

In mice and humans iNKT cells secrete large amounts of IFN-γ, IL-4, IL-2, IL-5, IL-10, IL-13, GM-CSF and TNF-α within minutes after TCR stimulation (158, 234, 243), a property that distinguishes these cells from conventional T cells that acquire their ability to secrete cytokine after primary stimulation. IL-4 and IFN-γ protein can be detected in activated, but not in resting iNKT cells (149). However abundant mRNA transcript for IL-4 and IFN-γ are present in resting iNKT cells (244, 245). Indeed iNKT cells activate IL-4 and IFN-γ transcription already during thymic development and this constitutive cytokine mRNA expression may allow the rapid cytokine production and secretion. In addition to the prompt cytokine secretion, iNKT cells have a strong cytolitic activity as they release perforin and granzymes and express membrane-bound members of the TNF family (150).

iNKT cells show a very peculiar kinetics of cytokine production and expansion upon activation (246). In fact, as demonstrated by αGalCer *in vivo* administration, shortly after their activation, iNKT cells produce mainly IL-4. Anyway at later time points cytokine production shifts toward a Th1 profile. Within two hours from the activation, iNKT cells start to downregulate their TCR as well as NK1.1 marker. TCR downregulation reaches the peak after 8-12 hours from stimulation but after 24 hours TCR is upregulated and iNKT cells can be detected again by flow cytometry (246). As consequence of their activation, iNKT cells undergo to a robust cellular expansion. This expansion peaks at day three upon αGalCer administration and it is follows by a contraction phase after 7-14 days (246).

Thanks to their ability to upregulate costimulatory molecules and to produce cytokines, iNKT cells can give rise to a network of activation involving cells from innate and adaptive immune system compartment. For instance IFN-γ and IL-2 produced by iNKT cells, in combination with IL-12 produced by DCs can lead to activation of NK cells and memory $CD4^+$ and $CD8^+$ T cells (247-250). This activation seems to increase IFN-γ production and cytotoxicity by these cells. Moreover, through the production of IFN-γ, iNKT cells may also contribute to the activation of

macrophage (251). On the other hand, IL-4 secretion and likely CD40/CD40L interaction, mediate B cell activation and immunoglobulin production (247, 252). In addition, when activated during mice immunization with protein antigens, iNKT cells were shown to help antigen specific antibody production, even in the absence of conventional T cells (253).

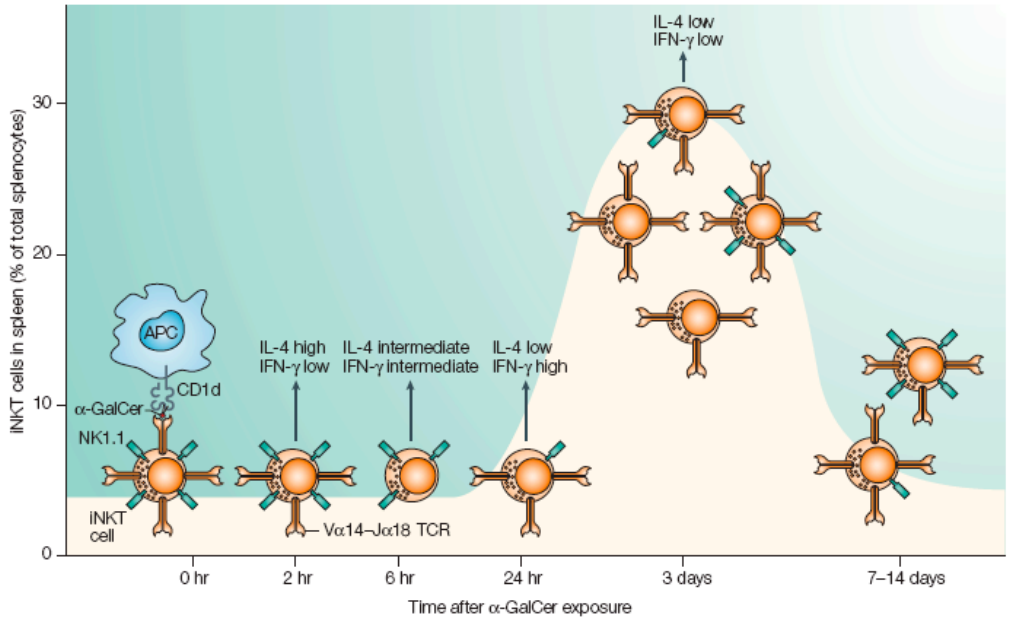


Figure X: The *in vivo* response of iNKT cells to stimulation with αGalCer.
Soon after administration of αGalCer to mice, this reagent binds CD1d at the cell surface of APCs and is presented to iNKT cells. Within hours, iNKT cells become activated and start to secrete IL-4 and IFN-γ. αGalCer-activated iNKT cells also rapidly downregulate cell-surface-expressed TCR and NK1.1, rendering these cells undetectable with flow-cytometric reagents that bind these markers. TCR expression levels return to almost normal at 24 hours (hr) after injection of αGalCer. iNKT cells then rapidly proliferate, clonally expanding 10- to 15-fold in the spleen and less extensively in other organs. Most iNKT cells subsequently die, to maintain homeostatic numbers. The cell-surface expression levels of NK1.1 remain suppressed for an extended period. Early (2 hr) during the response to αGalCer, iNKT cells mainly produce IL-4. At 24 hr, these cells mainly produce IFN- γ, and at 3 days after injection of αGalCer, when iNKT-cell numbers are maximal, these cells produce few cytokines. Modified from Van Kaer (246).

2.5.9 Signaling pathways involved in iNKT cell function

As discussed for iNKT cell development, important signaling pathways, arising from the CD1d mediated TCR triggering, have been demonstrated to be crucial in the function of this peculiar T cell subset.

TCR engagement activates PKCθ, which relays signals to downstream effectors, and PKCθ was shown to be required not only for the development but also for the function of iNKT cells (214). In conventional T cells, the Tec kinase Itk plays a significant role in signaling downstream the TCR by mediating the activation NFAT and AP-1 transcriptional factors. The lack of Itk in iNKT cells leads to a severe reduction in both Th1 and Th2 cytokine production upon *in vivo* activation (223).

NF-κB and NFAT as well, which are two of the most relevant transcriptional factors activated following TCR triggering, have been involved in the regulation of iNKT cell function. Indeed in the absence of NF-κB, iNKT cells are not able to produce detectable levels of neither IL-4 nor IFN-γ upon *in vivo* and *in vitro* activation (214). The member of the NFAT family NFAT2 seems to regulate Th2 but not Th1 responses in iNKT cells, as suggested by the increased nuclear translocation upon iNKT cell activation and by the enhanced IL-4 production after iNKT cell infection with a retrovirus construct expressing NFAT2 (254).

In addition, iNKT cells lacking the Th1 master regulator T-bet, as expected exhibited a decreased IFN-γ production (221). By contrast GATA-3, which in conventional T cells serves as a direct transcriptional factor for several Th2 cytokine genes, was shown to be required for the *in vivo* production of both IL-4 and IFN-γ production (255).

2.5.10 iNKT cells in antimicrobial immunity

iNKT cells contribute to antimicrobial host responses in bacterial, parasitic, viral and fungal infection (240).

The protective role of iNKT cells in response to bacteria was shown in an acute pneumonia model following *Streptococcus pneumoniae* infection (256). Upon infection with *S. pneumoniae*, mice lacking iNKT cells displayed more bacteria in the lung and died within several days from the infection. iNKT cells seem also to be

involved in protective responses toward some kind of parasites, as suggested by the increases susceptibility to visceral *Leishmania major* infection of iNKT cell null mice (257). iNKT cells also participate in the responses to viruses, although differently from bacteria and parasites, viruses contain only host lipids. In a skin infection model with a virulent strain of herpes simplex virus type 1 (HSV-1), it was shown that mice lacking iNKT cells are more susceptible to infections and present a delayed virus clearance (258). Although another group was not able to reproduce these data using a less virulent HSV-1 strain (259), the protective role of iNKT cells in HSV infection was further supported by the evidence that iNKT cell null mice are more susceptible to genital HSV-2 infection (260). Upon infection, several viruses can downregulate the expression of CD1d. This finding suggests the existence of an immune evasion mechanism that prevents CD1d mediated antigen presentation to iNKT cells (240).
In some microbial infection model, iNKT cells were anyway demonstrated to be not protective, but on the contrary to exert a detrimental activity, as in the case of *Chlamydia trachomatis* infection (261). The proposed mechanism indicates that iNKT cells enhance *Chlamydia* infection through the augmentation of Th2 responses, although for unknown reason, in other infections they can promote Th1 responses and pathogen clearance.

2.5.11 iNKT cells in antitumor immunity

iNKT cells can potently promote tumor rejection in murine models that use αGalCer as stimulus, but they also contribute to the natural antitumor immune surveillance in the absence of exogenous stimulation.
αGalCer was initially purified from marine sponges on the basis of its antitumor properties (166). Antitumor effects of αGalCer have been observed against different tumors and their metastasis, including melanoma, carcinomas and lymphomas (166) (262-264). The mechanism hypothesized for this antitumor activity relays on capacity of the activated iNKT cells to induce, by their IFN-γ production and CD40L upregulation, the production of IL-12 by DCs and the activation of NK and CD8 cytotoxic T lymphocytes that function as directs antitumor effectors (263-266).
Studies using chemical mutagenesis with methylchlorantrene (MCA) suggested that iNKT cells contribute to natural tumor immunosurveillance, in the absence of

exogenous stimulation by αGalCer (267). Natural host immunity against MCA-induced sarcoma requires endogenous IL-12 production, early IFN-γ produced by iNKT cells and other cells, such as NK cells and CD8 T cells (268). Tumor rejection is then mediated by NK cells and CD8 T and is perforin-dependent, whereas iNKT cells are required but exert their antitumoral activity in a perforin-independent manner. Moreover, adoptive transfer experiments demonstrated that CD1d expression was essential, thus suggesting that recognition of CD1d presented antigens may be required for the natural antitumoral activity of iNKT cells (269).

Additional evidences that strengthen the role of iNKT cells in antitumoral immunity derive from the observation of numerical deficiencies of iNKT cells and their loss of IFN-γ production in patients with advanced cancers (e.g. advanced prostate cancer or myelodisplastic syndrome) (270, 271). In advanced prostate cancer patients, iNKT cells were impaired in their ability to secrete IFN-γ, but not IL-4, thus generating a Th2 bias that was hypothesized to mediate the suppression of antitumor responses (270).

2.5.12 iNKT cells in autoimmunity

In some autoimmune disease model, iNKT cells help to maintain tolerance to self-antigen and thereby prevent autoimmune disease. However in other models iNKT cells can also play a pathogenic role.

A numerical deficiency of iNKT cells was found in nonobese diabetic (NOD) mice, a mouse strain that spontaneously develops a form of autoimmune diabetes similar to human type I diabetes (159, 272). Moreover CD1d deficient NOD mice, thus lacking iNKT cells, had earlier diabetes onset, higher disease penetrance, and more severe disease, suggesting that the deficiency of iNKT cells might be linked to the development of diabetes in NOD mice (273-275). In addition, the development of diabetes in NOD mice was ameliorated by adoptive transfer of normal iNKT cells (276) and by the transgenic expression of the invariant Vα14Jα18 TCR (277). Protection from diabetes conferred by iNKT cells was associated to a Th2 shift within pancreatic islet since IL-4 is as a key mediator of the immunoregulation induced by iNKT cells (276). However the mechanism of action and the target cells of IL-4 have not been determined. In addition to a role in natural course of type I diabetes,

stimulation of iNKT cells by αGalCer in NOD mice prevented the onset of diabetes and prolonged the survival of pancreatic islet transplanted into newly diabetic NOD mice (272, 273, 275, 278). This protection from diabetes was associated with suppression of both T and B cell autoimmunity and IFN-γ production, and the generation of tolerogenic islet autoantigen-specific T cells with a protective cytokine production profile (272, 278).

iNKT cells were found to be decreased in number also in patients with multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS) (279). Other data, linking iNKT cells to MS, derive from EAE, an animal model of MS that is induced by immunizing susceptible rodent strains with CNS antigens. However, the results obtained from αGalCer treatment of EAE have likewise generated conflicting results. Injections of αGalCer were found either to prevent disease (280, 281), to have no effect (282, 283) or, in one protocol, to accelerate disease (280). This could be due to differences in the models used, which include different antigens, different mouse strains and different timing of αGalCer injection (246, 279). Similar to the NOD model of diabetes, when αGalCer was effective, it usually prevented EAE by shifting the balance from a pathogenic Th1 towards a protective Th2 response to CNS antigens (280, 281). In keeping with this finding, the glycosphigolipid antigen OCH, which favors IL-4 production by iNKT cells, was more effective than αGalCer in preventing EAE in C57BL/6 mice (283).

Similarly, OCH was effective also in protecting mice against the development of collagen-induce arthritis and disease protection correlated with the capacity of OCH to promote the production of Th2 cytokines by iNKT cells and to bias collagen-specific T cells for production of Th2 cytokines (246).

iNKT cells have been hypothesized to play a pathogenic role in NZB/NZW mice, a strain that develops lupus spontaneously, because some studies showed that the transfer of activated iNKT cells in NZB/NZW mice induced an autoimmune-like inflammation (284). In addition hyper reactive iNKT cells were found to accumulate in aging NZB/NZW mice (285), and suggested to help B cell in the production of anti-DNA antibodies (284). However, studies on CD1d deficient lupus-prone mice have not yielded concordant results (286-288) and injection of αGalCer ameliorated or aggravated the disease (289).

2.5.13 iNKT cells in primary immunodeficiencies

X-Linked lymphoproliferative syndrome (XLP) is a rare inherited immunodeficiency, characterized by a high susceptibility to Epstein–Barr virus (EBV) infection that triggers a severe infectious mononucleosis, with the features of a hemophagocytic lymphohistiocytosis (HLH) in 60% of cases (290). Patients who do not develop HLH could present other aggressive lymphoproliferative and inflammatory manifestations such as lymphoma, lymphoid vasculitis and colitis. XLP is caused by mutation in SAP (SLAM-associated protein) or in XIAP (X-linked inhibitor of apoptosis protein) genes. In 2005 two independent studies shown that SAP deficient mice and humans are devoid of iNKT cells (227, 228), thus revealing a pivotal role for SAP molecule in the development of this particular T cell subset as discussed above. In addition this finding suggested a role of iNKT cells in the successful immune responses against EBV infection.

XIAP-deficient patients do not show T-, B- or NK-cell lymphopenia, with the exception of iNKT cells, which are markedly reduced. The defect of iNKT cells in XIAP deficiency is different from that observed in SAP deficiency since few iNKT cells are clearly detected in the blood of XIAP-deficient patients while XIAP-deficient mice have normal iNKT cell numbers (290, 291). Indeed XIAP might be involved in the peripheral homeostasis and/or in the late stages of NKT cell development.

Moreover, in keeping with iNKT cell immunoregulatory role, it has been recently described the absence of this cellular population in another primary immunodeficiency associated with autoimmunity, the Omenn syndrome, characterized by the presence of autoreactive oligoclonal T cells and severe autoimmune manifestations (292).

3. SPECIFIC AIMS

The Wiskott-Aldrich Syndrome is a complex genetic disease, caused by mutation in the gene encoding for WASp, with a major involvement of the immune system. WAS patients are more susceptible to develop infection and malignancies than healthy individual. WASp is expressed in all hematopoietic cells, where it regulates actin polymerization in response to signals arising at the plasma membrane. Although WASp deficiency impairs several cellular functions, thereby affecting both innate and adaptive immune response, its role in the biology of iNKT cells has never been defined. iNKT lymphocytes are important immunoregulatory elements involved in the control of pathogen infections and in tumor immunosurveillance. Thus alteration in iNKT cell compartment might contribute to the immune dysregulation of WAS.

The present thesis aims at characterizing such defect that could contribute to explain defective response to bacteria and high tumor incidence observed in these in WAS patients. Indeed, full comprehension of the alterations caused by the lack of WASp will allow a better evaluation of the efficacy of the current therapeutical approaches.

We therefore investigated i) the phenotype of iNKT cells isolated from WAS and XLT patients, ii) the phenotype and the tissue distribution of iNKT cells in *was*$^{-/-}$ mice, iii) the development of murine *was*$^{-/-}$ iNKT cells and iv) the effector function of murine *was*$^{-/-}$ iNKT cells.

4. MATERIALS AND METHODS

4.1 PATIENTS

Peripheral blood samples from patients and age matched HDs were obtained following standard ethical procedures in accordance with San Raffaele Scientific Institute Internal Review Board. Clinical phenotype of the patients is summarized in Table I in the Result Section.

4.2 MICE

C57BL/6 (B6) *was*$^{-/-}$ mice were kindly provided by K.A. Siminovitch. CD45.1 *was*$^{-/-}$ mice were generated in our facility. B6 CD45.2 wt mice were purchased from Charles River Laboratories. All mice, unless that specifically indicated, were from 8 to 12 weeks of age. Mouse studies were performed under the San Raffaele Institutional Animal Care and Use guidelines.

4.3 CELL PREPARATION AND FLOW CYTOMETRY

4.3.1 Human cells

PBMCs were purified from peripheral blood on Lymphoprep gradient (Axis-Shield PoC AS). For FACS detection of iNKT cells, PBMC were stained with: anti-CD3 (UCHT1) from BD Biosciences; anti-TCRVα24 (C15) and anti-TCRVβ11 (C21) from Immunotech. Alternatively for the measure human iNKT cells, PBMC were stained with anti-CD3 (UCHT1) and either αGalCer loaded human CD1d dimers or tetramers. Human CD1d dimers (DimerX I) were purchased from BD Biosciences while human CD1d tetramers were a gift of Dr. Badolato. For lipid loading CD1d dimers were incubated overnight with a 9 molar excess of αGalCer (Axxora) while human CD1d tetramers were already pre-loaded with αGalCer. Anti-WASp Ab was kindly provided by Prof. H. Ochs. TCR Vβ repertoire was performed on PBMCs from XLT and WAS patients using the IOTest® beta Mark from Beckman Coulter. All

flow cytometric staining were performed in FACS buffer (PBS 0,3% BSA 0,1% NaN_3).

4.3.2 Murine cells

For mouse studies, single cell suspensions were obtained from liver, spleen and thymus. Hepatic leukocytes were purified using a Percoll (Sigma-Aldrich) gradient. All the flow cytometric staining were performed in FACS buffer (PBS 0,3% BSA 0,1% NaN_3), unless that specifically indicated. Before antibody staining FcR was blocked using anti-CD16/32 mAb (BD Bioscience). The following mAbs were used for iNKT cell staining: anti-B220 (RA3-6B2) and anti-CD8a (53-6.7) from BD Biosciences; anti-CD3 (17A2) fromBioLegend and CD1d tetramers from Proimmune. For lipid loading, CD1d tetramers were incubated overnight with a 12 molar excess of αGalCer (Axxora).

For TCR Vβ analysis cells were stained with anti-TCR Vβ mAbs from TCR Vβ Screening Panel (BD Bioscience). Screening of TCR Vα was performed using anti-TCR Vα2 (B20.1), anti-TCR Vα3.2 (RR3-16) and anti-TCR Vα 8.3 (B21.14) mAbs, all from BD Bioscience. The following mAbs were used for the study of iNKT cell phenotype: anti-CD4 (RM4-5); anti-TCR Vβ7 (TR310); anti-TCR Vβ8 (F23.1); anti-CD44 (IM7); anti-CD25 (PC61); anti-CD69 (H1.2F3), all from BD Bioscience; anti-CD49b (DX5) and anti-NK1.1 (PK136) from BioLegend. Anti-CD1d (1B1); anti-CD8 (53-6.7); anti-CD19 (1D3) and anti-CD11c (HL3) mAbs, used for the analysis of CD1d expression, were purchased from BD Bioscience. For Bone Marrow Chimera studies it was utilized anti-CD45.2 (104) mAb from BD Biosciences. For the evaluation of IL-2Rβ chain receptor was used anti-CD122 (5HA) mAb from BD Biosciences. L363 Ab, used for the evaluation of the aGalCer-CD1d complexes was kindly provided by Steven Porcelli. Intracytoplasmic WASp staining was performed, following surface staining, using the Cytofix/Cytoperm kit (BD Biosciences). Anti-WASp Ab was kindly provided by Prof. H. Ochs. For the evaluation of intracellular cytokine production, cells were fixed in 1% paraformaldheyde and after the fixation were permeabilized by 15 minute incubation in FACS buffer supplemented in 0,5% Saponin (Sigma-Aldrich). Permeabilized cells were incubated in FACS buffer with 0,5% Saponin and 2% rat serum in the presence of the following mAbs: anti-IFNγ

(XMG1.2) and anti-IL-4 (11B11) (BD Biosciences). After washing, cells were acquired on a FACS CANTO (BD Biosciences) and analyzed with FlowJo Software (TreeStar Inc.).

For iNKT cell sorting, splenic $B220^-$ cells were purified by immunomagnetic techniques using anti-CD45R (B220) magnetic beads (Miltenyi Biotech) to enrich iNKT cell fraction. $B220^-$ cells were stained with anti-TCRβ mAb and PBS57 loaded CD1d tetramers. CD1d tetramers loaded with PBS57 were kindly provided from NIH Tetramer Core Facility. iNKT cells ($TCR\beta^+CD1d$ $tetramers^+$) were then sorted by MoFlo Fluorescence Activated Cell Sorter (Dako Cytomation).

4.4 *IN VITRO* EXPANSION OF HUMAN Vα24 Vβ11 iNKT CELLS

The protocol for the *in vitro* expansion of human Vα24 Vβ11 iNKT cells was previously described by Rogers P. et al (293). Briefly for the initial stimulation PBMC were cultured at $1x10^6$ cells/ml in a 24-well plate in X-VIVO™ 15 (Lonza) supplemented with 10% normal human serum, 2mM glutamine, 100 IU/ml penicillin + 100 IU/ml streptomycin (Invitrogen). αGalCer (Axxora) was added at the start of culture and rhIL-2 100 U/ml (Chiron) was added 16 hours later. For restimulation, remaining cells were cultured at $2x10^5$-$1x10^6$ cells/ml with $1x10^6$ cells/ml irradiated (3000 Rads) αGalCer pulsed PBMCs. PBMCs were pulsed in complete medium supplemented with αGalCer 100ng/ml for 4-5 hours at 37°C before irradiation. rhIL-2 100 U/ml was added one day later. Cells were restimulated weekly for two times and then sorted by MACS using anti-Vα24 PE conjugated antibody and anti-PE microbeads (Miltenyi Biotec). After sorting $V\alpha24^+$ cells were stimulated three times with irradiated αGalCer pulsed PBMC and rhIL-2 before the evaluation of iNKT cell expansion. Cells were analyzed 10-11 days following the last stimulation.

4.5 GENERATION OF MIXED BONE MARROW CHIMERAS

BM lineage marker-depleted (lin^-) cells from CD45.2 B6 wt or *was*$^{-/-}$ mice were purified using the murine hematopoietic progenitor enrichment kit (Stem Cell Technologies). Recipient *was*$^{-/-}$ mice (CD45.1) were irradiated (900 rad) before receiving i.v. $2.5x10^5$ lin^- cells. Reconstitution was monitored by flow cytometry on circulating cells. Mice were sacrificed 7-9 weeks after transplantation for the analysis of iNKT cells in thymus and liver. Thymocytes were depleted of $CD8^+$ cells by anti-CD8 magnetic beads (Miltenyi Biotec) to enrich iNKT cell fraction.

4.6 ACTIVATED CASPASES ASSAY

To measure the level of activated Caspases, $1x10^6$ hepatic leukocytes or thymocytes depleted of $CD8^+$ cells were incubated for 20' at 37°C with PBS supplemented with CaspACE FITC-VAD-FMK in situ marker (5μM, Promega Corporation). After the incubation cells were washed and stained for flow cytometric analysis of iNKT cells as described in CELL PREPARATION AND FLOW CYTOMETRY paragraph.

4.7 EVALUATION OF HOMEOSTATIC PROLIFERATION BY BrdU LABELLING

For the evaluation of 5-bromo-2-deoxyuridine (BrdU) incorporation, 5 weeks old mice were intraperitoneally injected with 100 μl of a 10 mg/ml solution of BrdU (Sigma) in PBS at the onset of the experiment. At the same time, BrdU (0,8 mg/ml) was placed in drinking water supplemented with 5% saccarose (w/v). This water was changed following 3 days from the beginning of the experiment. At day 6 mice were sacrificed and cells were stained with a BrdU flow kit (BD Bioscience) following the manufacturer's instruction for flow cytometric analysis.

4.8 *IN VITRO* CYTOKINE STIMULATION

IL-15 induce proliferation was evaluated in thymocytes depleted of $CD8^+$ cells by anti-CD8 magnetic beads (Miltenyi Biotec). After CD8 depletion, thymocytes were labeled with CFSE (Molecular Probes). CFSE labeling was performed by incubating the cells with CFSE 1,5 μM for 8' RT and then adding an equal volume of FCS to block CFSE staining. Labeled thymocytes were then plated in 24-well culture plates at the concentration of $2x10^6$ cells/well in complete RPMI 1640 medium (RPMI 10% FCS, 2mM glutamine, 100 IU/ml penicillin + 100 IU/ml streptomycin) containing recombinant human IL-15 (R&D System) at 100ng/ml. Each condition was done in duplicate, with cells from the two wells pooled for analysis after 4 days in culture.

4.9 FUNCTIONAL CHARACTERIZATION OF *was*$^{-/-}$ iNKT CELLS

4.9.1 *In vivo* activation and cytokine production

wt and *was*$^{-/-}$ mice were injected i.v with 1 μg of αGalCer in PBS or not injected as controls. To measure the *in vivo* IL-4 and IFN-γ production, blood samples were collected after 3, 6, 12 and 24 hours. Serum cytokine levels were measured by Bio-Plex Technology (BIO-RAD) following manufacture's instruction.

To test the *ex vivo* IL-4 and IFN-γ intracellular production by liver iNKT cells, mice were injected i.v with 1 μg of αGalCer in PBS or not injected as controls. Mice were then sacrificed 45' after αGalCer injection and livers collected. IL-4 and IFN-γ production by hepatic iNKT cells was determined by intracellular FACS staining.

4.9.2 *In vivo* iNKT cell expansion following αGalCer stimulation

Mice were injected i.v with 1 μg of αGalCer in PBS or not injected as controls. To evaluate iNKT cell expansion, wt and *was*$^{-/-}$ mice were sacrificed at day 3, 7 and 11 after the injection and livers were collected. Absolute numbers of live hepatic leukocyte were determined and iNKT cell expansion was measured by Flow Cytometry.

4.9.3 Mice immunization and antigen-specific Ab titer measurement

Mice were immunized with TNP-KLH antigen (Biosearch Technologies Inc, 20 μg per dose). Immunizations were performed on day 0 and 15 and groups of 5 mice were injected i.p. with antigen in 100 μl of PBS alone, with αGalCer (1 μg per dose) or with Lypopolysaccharide (LPS) from E.Coli 055:B5 (25mg/dose, Sigma Aldrich). Circulating antigen specific antibodies were titrated by endpoint ELISA. Serial 1:3 dilutions of sera were plated onto ELISA plate (Nunc) previously coated with TNP-KLH antigen. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Aldrich) followed by the pNPP substrate (Sigma Aldrich). Antibody titers are expressed as reciprocal dilution giving an $OD_{405} > OD_{405}$ plus 3SD. Blanks consistently displayed $OD_{405} < 0,1$ and < 10% variability.

4.9.4 *In vitro* activation of iNKT cells by αGalCer loaded DCs

Splenic iNKT cells from wt and *was*$^{-/-}$ mice were sorted by FACS as described in CELL PREPARATION AND FLOW CYTOMETRY section. To isolate wt and *was*$^{-/-}$ DCs, the cellular fraction containing DCs was first enriched by OptiPrep® Density Gradient Medium (Axis Shield). DCs were then purified by immunomagnetic techniques using anti-CD11c magnetic beads (N418, Miltenyi Biotech) and DC purity was assessed by Flow Cytometry. For αGalCer pulsing, DCs were incubated overnight at 37°C with αGalCer 100 ng/ml. Following washing, αGalCer pulsed DCs were seeded at 3,5 x 10^5 cells/well in a 96-well plate in RPMI 1640 supplemented with 10% FBS, glutamine and penicillin/streptomycin. iNKT cells were co-culture with DCs in a 1:1 ratio. After 48 hours of co-culture cytokine production was evaluated on supernatants by Bio-Plex Technology (BIO-RAD) following manufacture's instruction.

4.9.5 αGalCer presentation by Bone Marrow derived DCs (BM DCs)

For the generation of BM DCs, bone marrow cells from wt and *was*$^{-/-}$ mice were plated in 100 MM plate (Sterilin Limited) at 4 x 10^5 cells/ ml in IMDM supplemented with 10% FCS, 2mM glutamine, 100 IU/ml penicillin + 100 IU/ml streptomycin, β Mercaptoethanol 50 μM and a 30% of supernatant from a granulocytes-macrophage

colony stimulating factor (GM-CSF)-expressing cell line (BV06) (20-30 ng/ml GM-CSF/ml). Every three days cells were split and medium replaced. BM DC differentiation was evaluated at day ten by Flow Cytometry. Pool of BM DCs from wt and *was*$^{-/-}$ mice were incubated for 18 hours at 37°C with IMDM complete medium supplemented with αGalCer 100 ng/ml. αGalCer presentation was analyzed by Flow Cytometry using L363 Abs.

4.9.6 *In vitro* cytokine production by stimulated iNKT cells

To enrich the iNKT cell containing fraction, CD90^{+} cells were purified by anti-CD90.2 magnetic beads (Miltenyi Biotech) from total wt and *was*$^{-/-}$ splenocytes. Following isolation, CD90^{+} cells were plated at 1x 10^{6} cells/ well (48 well plate) in complete RPMI 1640 medium (RPMI 10% FCS, 2mM glutamine, 100 IU/ml penicillin + 100 IU/ml streptomycin) supplemented with 12-O-tetradecanoylphorbol-13-acetate (TPA, 50 ng/ml) and ionomycin (1μg/ml). After 1 hour of incubation at 37°C, Brefeldin A was added and cells were incubated for another hours. Following incubation cells were extensively washed and intracellularly stained for IFN-γ and IL-4 production by FACS.

To test cytokine production upon stimulation with anti-CD3 and anti-CD28 mAbs (BD Bioscience), splenic iNKT cells from wt and *was*$^{-/-}$ mice were sorted by FACS as described in CELL PREPARATION AND FLOW CYTOMETRY section. iNKT cells were then put in culture at 5 x 10^{5} cells/well in a 96-well round bottom plate in RPMI 1640 supplemented with 10% FBS, glutamine and penicillin/streptomycin in the presence of immobilized anti-CD3 mAb (2μg/ml) and soluble anti-CD28 mAb (2μg/ml) or with TPA (50 ng/ml) and ionomycin (1μg/ml). Following 48 hours of stimulation at 37°C, supernatants were collected and cytokines measured by Bio-Plex Technology (BIO-RAD) following manufacture's instruction.

4.9.7 Measurement of TCR Avidity

For the evaluation of iNKT cell TCR avidity, total splenocytes were incubated for 30' with different concentration of PBS57-loaded CD1d tetramers (0.25, 0.5, 1, 2.5 and 5

nM), with anti-B220 and anti-CD3 mAbs in FACS buffer. TCR avidity was measured by Flow Cytometry.

4.10 STATISTICAL ANALYSIS

All the data were analyzed with a two-tailed Mann-Whitney test. P values of less than 0,05 were considered significant.

5. RESULTS

5.1 CHARACTERIZATION OF Vα24 Vβ11 iNKT CELLS FROM WISKOTT-ALDRICH SYNDROME PATIENTS

5.1.1 Lack of Vα24 Vβ11 iNKT cells in WAS, but not in XLT patients

To unravel the potential requirement of the Wiskott-Aldrich Syndrome protein (WASp) by human Vα24 Vβ11 iNKT cells, we evaluated what happens to iNKT cells in the case of WASp deficiency. To this aim, we totally collected and examined peripheral blood samples from three patients with a mild clinical score (score 1-2, XLT), six patients with severe WAS (score 3-5) and 13 age matched HD controls. The molecular and clinical features of all the patients analyzed are reported in Table I. The flow cytometric analysis of the invariants Vα24 and Vβ11 chains revealed, as displayed in the representative density plot of Figure 1A, that iNKT cells are nearly undetectable in WAS patients with a severe clinical phenotype. On the contrary, XLT patients presented a detectable number of Vα24 Vβ11 iNKT cells. The graph summarizing the results from all the patients (Figure 1B), shows that in WAS patients the iNKT cell population is almost completely absent, while in XLT patients the level of iNKT cells, even in the lower range, is not significantly different from HD controls. Thus impairment in the amount of circulating iNKT cells correlates with severity of the disease.

As alternative approach, human iNKT cells can be detected by αGalCer loaded CD1d dimers/tetramers. To confirm the specificity of anti-TCR-Vα24 and anti-TCR-Vβ11 mAbs in detecting human iNKT cells, we compared $Vα24^+$ $Vβ11^+$ cells with the population detected by αGalCer-loaded hCD1dimers. In parallel, to verify unspecific binding of dimers, the sample was labeled with unloaded CD1d dimers. As displayed in Figure 2A, consistently with previously published data (227, 294) the two technical approaches detected comparable percentages of iNKT cells in HD controls. Furthermore, the staining of XLT and WAS patients with CD1d tetramers (Figure 2B) confirmed the absence of iNKT cells in severe WAS as well as the presence of a small

iNKT cell population in XLT patients. Altogether these data demonstrate that WAS, but not XLT patients lack Vα24 Vβ11 iNKT cells.

Patient	Age[a]	Mutation Type	gDNA mutation	Protein Change	Clinical Score[c]	Thrombo-cytopenia	T cell lymphopenia	Infections	Tumor
WAS 1	26 years	Splice Intron 9	IVS9 + 2 del tgag	ND	WAS (5)	Yes	No	Severe HSV, pneumonia	Yes
WAS 26	21 months	Missense Exon 4	431 G>A	E133K	XLT (2)	No[d]	No	Recurrent fevers	No
WAS 27	22 years	Missence Exon 1	150T>C	L39P	XLT (2)	Yes[d]	No	Recurrent influenza	No
WAS 28	29 years	Splice Intron 2	IVS2 + 4 T>C	ND	WAS (3)	Yes	No	Bronchiectasis, mild recurrent infection	No
WAS 29	6 months	Nonsense Exon 1	155 C>T	R41X	WAS (3)	Yes	Yes	Recurrent upper respiratory tract infections, otitis media andurinary tract infections	No
WAS 30	19 years	Missense Exon 12	1487 G>A	D485N	XLT (2)	Yes	No	Few upper respiratory tract infections	No
WAS 31	13 months	Splice	NR[b]	No expression	WAS (3)	Yes[e]	Yes	Mild recurrent infections	No
WAS 32	32 months	Insertion Exon 10	C 1238-1239 Ins C	P402X949	WAS (5)	Yes[e]	No	Persistent CMV	No
WAS 33	5 years	Entire gene missing	Entire gene missing	No expression	WAS (4)	Yes[e]	No	Persistent CMV	No

Table I. Molecular and clinical data of WAS patients

del, deletion; IVS, intervening sequence (intron); ND, not determined; NR, not reported.
[a]Age refers to the time of blood sampling.
[b]Mutation is going to be published separately.
[c]Disease score is given according to the classification reported previously (4)
[d]Splenectomized.
[e]Undergoing platelet transfusion.

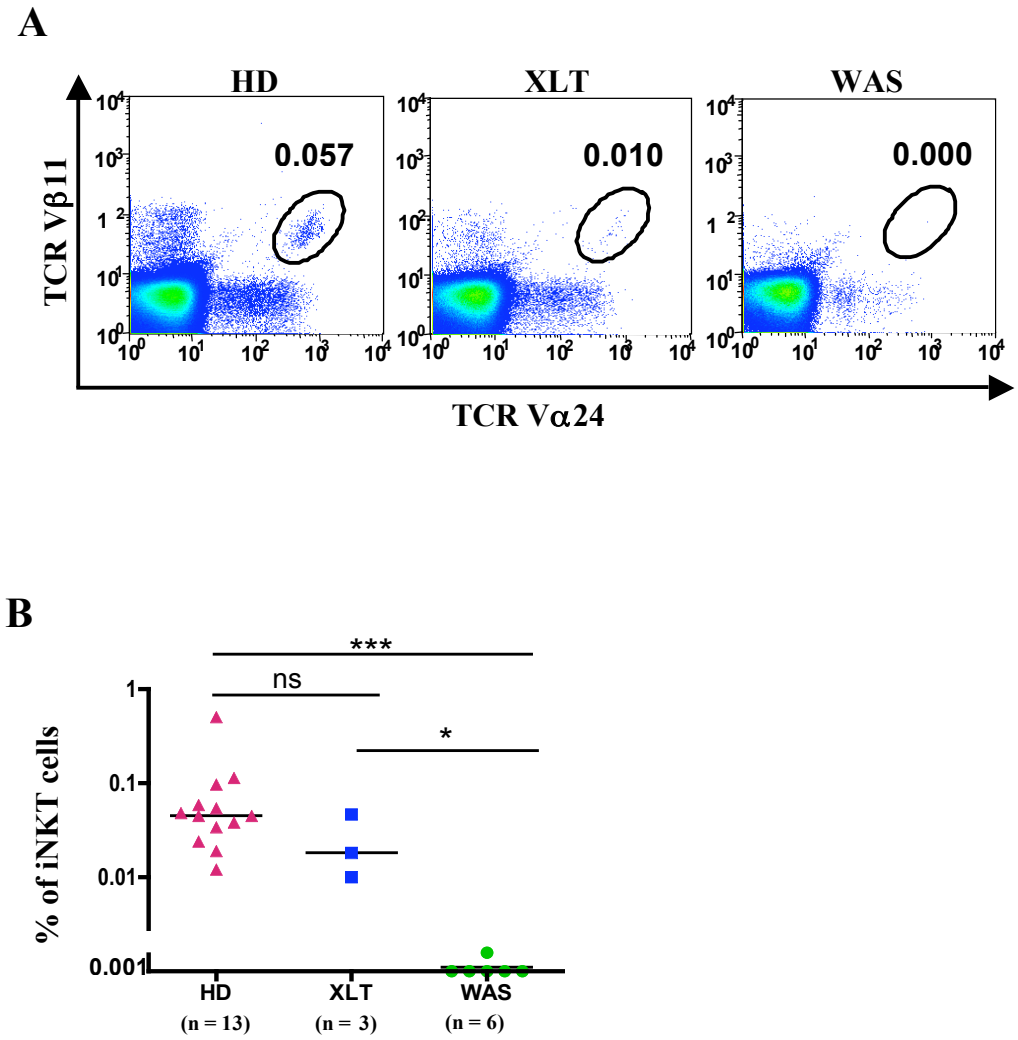


Figure 1: Frequency of iNKT cells in XLT and WAS patients. (A) Representative flow cytometric analysis of peripheral blood iNKT cells from an age matched Healthy Donor control (HD), an XLT patient (XLT) and a WAS patient (WAS). Cells in the density plots are gated on $CD3^+$ cells. Percentages of iNKT cells ($TCRV\beta11^+TCRV\alpha24^+$) are indicated. (B) Frequency of iNKT cells from 13 controls, 3 XLT and 6 WAS patients. Bars represent the median value of each group. ns = $p>0.05$, * = $p<0.05$ and *** = $p<0.001$.

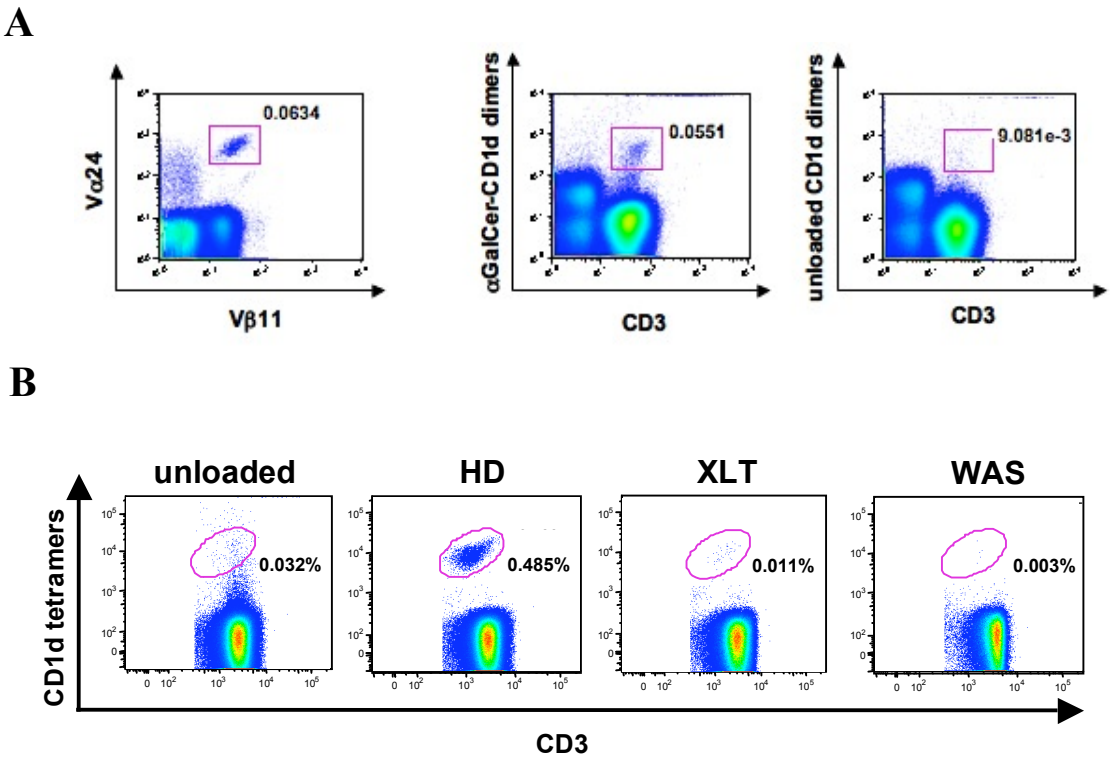


Figure 2: iNKT cell detection with αGalCer loaded CD1d dimers/tetramers. (A) Comparison of iNKT cells stained with anti-Vα24 and anti-Vβ11 Abs or with αGalCer CD1d dimers. PBL from the same HD control were stained with anti-Vα24 and anti-Vβ11 Abs or with anti-CD3 Ab and CD1d dimers loaded with αGalCer or unloaded as control. (B) Representative FACS analysis of frozen PBL stained with anti CD3 Ab and CD1d tetramers unloaded (background control) or loaded with αGalCer. Cells in the plot are gated on $CD3^+$ cells.

5.1.2 Presence of all TCR Vβ families in T lymphocytes of WAS patients

The complete absence of Vα24Vβ11 double positive T cells in WAS patients, raises the hypothesis that the lack of WASp can also impact the usage of some TCR Vα and Vβ chains by conventional T lymphocytes. To address this issue we analyzed by flow cytometry the TCR Vβ repertoire in the following patients: WAS1 (score 5), WAS 31 (score 3), WAS33 (score 4), WAS28 (score 3) and in one XLT case, WAS30. The analysis of 24 TCR Vβ families was performed on frozen PBMCs obtained from WAS patients (Table II). We were unable to detect any significant abnormalities in the TCR Vβ gene usage by WASp deficient T cells, indeed $CD3^+$ cells expressed all Vβ families. Nonetheless some TCR Vβ families were found expanded in comparison to the healthy donor range, probably due to concurrent infections, which are usually frequent in these immunodeficient patients.

Moreover, we measured the percentage of T ($CD3^+$) cells expressing either Vα24 or Vβ11 in XLT, WAS and normal donors (Figure 3). The frequency of T cells expressing either TCR Vα24 or Vβ11 in severe WAS patients lacking iNKT cells was, although variable, in the range of HDs, suggesting that these TCR V regions could be utilized without specific impairments by mainstream T cells of WAS patients. These data indicate that the absence of normal WASp causes a strong defect in the frequency of circulating iNKT cells in spite of minor alteration of conventional T lymphocytes.

Vbeta	HD[a] Mean[b]	HD[a] Min[b]	HD[a] Max[b]	WAS 1 %[b]	WAS 28 %[b]	WAS 30 %[b]	WAS 33 %[b]
Vb1	3,53	1,89	11,7	3,18	1,73	8,31	0,8
Vb2	8,30	4,03	23,48	4,30	2,44	6,87	1,19
Vb3	4,68	0,52	15,71	0,15	0,31	1	0,54
Vb4	1,91	0,79	3,26	0,07	1,19	1,05	0,22
Vb5.1	5,45	3,19	14,93	3,34	1,26	3,98	0,56
Vb5.2	1,33	0,49	4,98	1,24	4,86	1,63	1,17
Vb5.3	1,08	0,37	2,98	0,03	1,82	5,08	0,67
Vb7.1	2,56	0,64	20,01	0,65	3,9	2,09	0,38
Vb7.2	1,47	0,05	5,45	1,46	1,35	3,29	0,22
Vb8	4,68	2,26	29,47	6,09	3,93	8,52	0,95
Vb9	3,13	1,1	9,3	0,28	4,55	1,55	0,2
Vb11	1,04	0,25	5,11	0,25	2,24	3,56	0,49
Vb12	1,66	1	4,76	1,89	1,84	1,07	1,10
Vb13.1	3,83	1,62	8,16	2,02	6,07	2,23	0,23
Vb13.2	2,80	0,8	5,28	0,27	3,37	2	1,29
Vb13.6	1,86	0,84	8,8	8,03	0,26	1,09	1,58
Vb14	3,49	1,33	8,03	0,95	1,88	4,63	13,44
Vb16	0,92	0,42	1,9	1,40	1,62	2,07	1,69
Vb17	5,15	2,28	12,61	1,62	2,37	5,16	0,54
Vb18	1,49	0,58	5,23	0,94	1,06	1,77	0,25
Vb20	2,52	0	9,73	0,9	0,79	0,94	1,10
Vb21.3	2,38	1,08	5,97	0,9	7,50	1,03	0,5
Vb22	3,84	1,99	9,89	2,02	1,37	2,88	14,57
Vb23	0,85	0,28	4,76	8,16	1,82	1,37	0,58

Table II. TCR Vbeta Repertoire of WAS patients

Min, minimum value obtained; Max, maximum value obtained.

[a] Healthy Donor values are reported in "Quick Reference Card" of TCR V beta Repertoire Kit by Beckman Coulter

[b] All the values represents the percentage of $CD3^+$ lymphocytes.

TCR Vbeta repertoire was performed on PBMCs from XLT and WAS patients using the IOTest® beta Mark from Beckman Coulter.

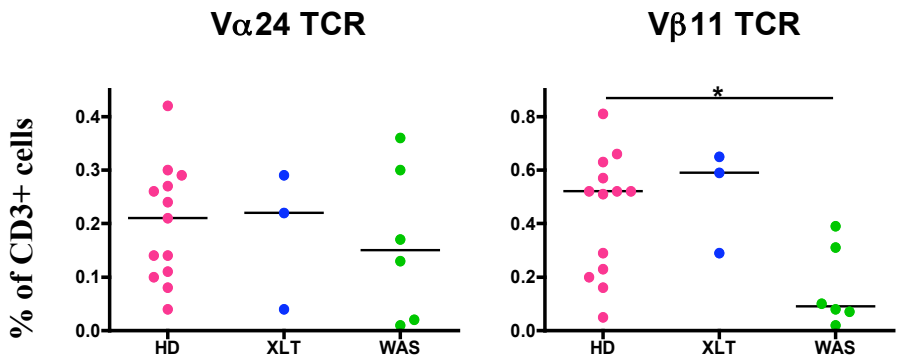


Figure 3: TCRVα24 and TCRVβ11 expression. TCRVα24 and TCRVβ11 expressions were analyzed on $CD3^+$ lymphocytes obtained from 13 healthy donors (HD), 3 XLT and 6 WAS patients. The graph shows the percentage of TCRVα24 and Vβ11 single positive cells excluding double positive ($TCRV\alpha24^+$ $V\beta11^+$) cells. Bars represent the median value of each group, * = $p<0.05$

5.1.3 Failure of WASp deficient iNKT cell expansion

Next we wanted to assess whether WASp deficiency causes alteration in iNKT cell function. To this aim we tried to expand *in vitro* iNKT cells from XLT and WAS patients, in order to perform, in case of a successful expansion, functional studies on this cellular population. To this end Vα24 Vβ11 iNKT cells were expanded from peripheral blood lymphocytes (PBL) of 6 Healthy Donor (HD) controls, 2 XLT (WAS26 and WAS 27) and 3 WAS (WAS1, WAS 28 and WAS 29) patients, by stimulating them several times with IL-2 and irradiated PBL pulsed with αGalCer (293), the specific iNKT cell stimulus, as shown in Figure 4A. As shown in Figure 4B, after several rounds of stimulation with αGalCer loaded irradiated PBL, iNKT cells from HD controls, XLT and WAS patients did expand and become measurable. Nevertheless the percentages of WASp deficient iNKT cells were between 0.2 and 9% of total expanded population. On the contrary normal iNKT cells were, with only one exception, the totality of the harvested population (Figure 4C). This result suggests that WASp deficiency alters iNKT cell ability to selectively expand in response to the specific stimulus αGalCer. Moreover, since the expansion of iNKT cells from the XLT/WAS group did not allow us to achieve enough iNKT cells, it was not possible to perform any functional assay on these cells.

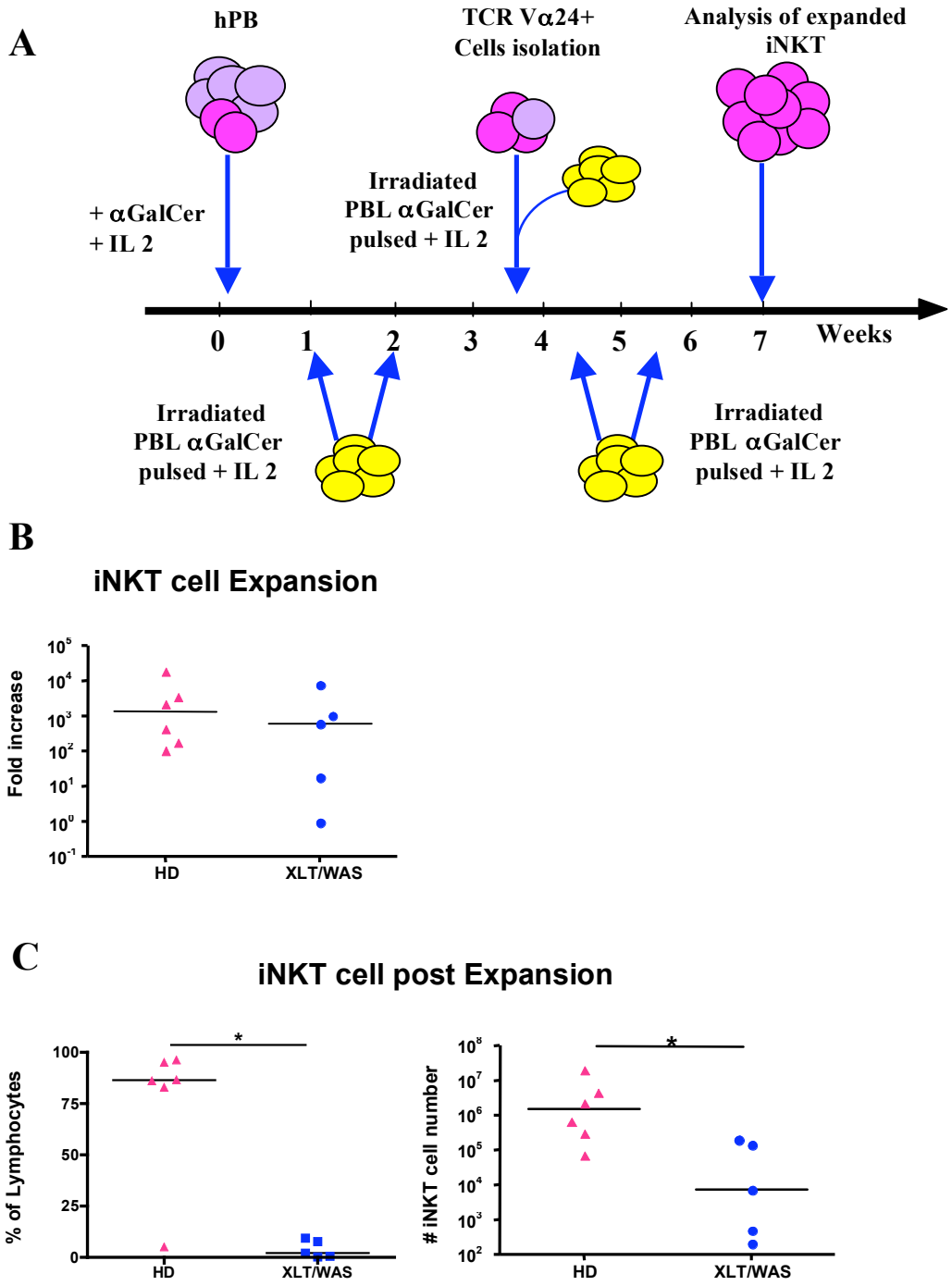


Figure 4: WASp deficient iNKT cell *in vitro* expansion. (A) PBL from WAS/XLT patients and normal donors were put in culture with αGalCer (100 ng/ml); IL-2 (100 U/ml) was added 16 hours later. αGalCer pulsed irradiated PBLs plus IL-2 were added after one and two weeks. Subsequently, TCR Vα24+ cells were sorted by MACS microbeads and were stimulated three times with irradiated PBL pulsed with αGalCer in presence of IL-2. (B) iNKT cells were expanded as described above, from PBL of 6 HD controls, 2 XLT and 3 WAS patients. Eight-eleven days from the last stimulation, cells were stained with anti-CD3, anti-TCR Vβ11 and anti-TCR Vα24 Abs to evaluate iNKT cell expansion. The graph depicts iNKT cell fold increase calculated as the ratio between total number of iNKT cell put in culture at time zero and after the expansion. (C) iNKT cell percentage on total lymphoid population (left graph) and absolute number (right graph) after the expansion. The graphs summarize the results of three independent experiments and bars represent the median value of each group, * = $p<0.05$

5.1.4 WASp is expressed by all human Vα24 Vβ11 iNKT cells

WASp is a cytosolic protein normally expressed by all hematopoietic cells, in which controls many important activities, as migration and effector function. In order to support the direct role of WASp in human iNKT cells, it was essential to confirm WASp expression on this peculiar T cell subset. Since Vα24 Vβ11 iNKT cells are present in human peripheral blood at very low and variable amounts (213), it was necessary to expand iNKT cells *in vitro*, as described above, before measuring WASp expression level by intracellular staining. The analysis of WASp expression in the expanded population revealed that, like all hematopoietic cells, the entire Vα24 Vβ11 iNKT cell population expresses WASp (Fig.1B). This finding confirms that, like in conventional T cells and in NK cells, WASp regulates crucial immune functions in iNKT cells.

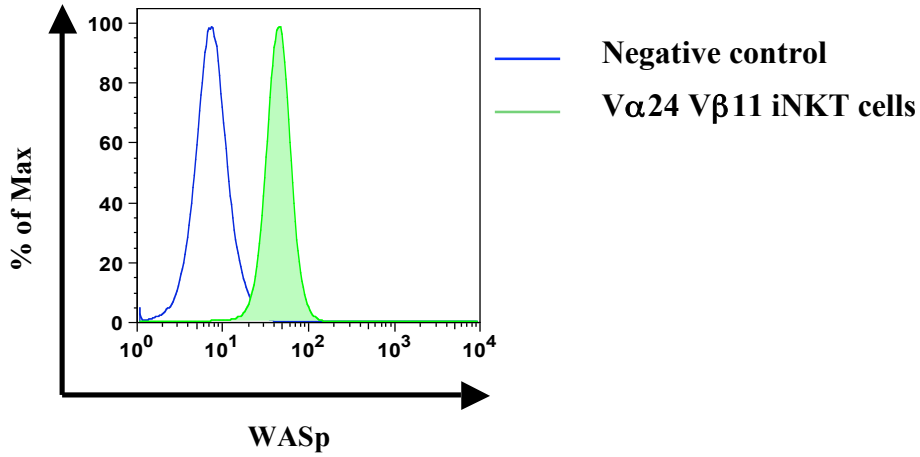


Figure 5: WASp expression by human Vα24 Vβ11 iNKT cells expanded in vitro. HD iNKT cells, *in vitro* expanded as described in Figure Legend 4A, were stained with anti-CD3, anti-TCR Vβ11, anti-TCR Vα24 and anti-WASp Abs eleven days from the last stimulation. The graph shows WASp expression by a negative control (blue empty histogram) and by $CD3^+$ Vα24 Vβ11 iNKT cells (green filled histogram) from a representative HD.

5.2 PHENOTYPICAL CHARACTERIZATION OF iNKT CELLS FROM *was*$^{-/-}$ MICE

5.2.1 WASp is expressed by murine iNKT cells

Given the paucity of material derived from WAS patients, we further investigated the role of WASp in the development and function of iNKT cells in *was*$^{-/-}$ mice (49). This mouse model recapitulates important immune system alterations of the human WAS. We first verified WASp expression also in normal murine iNKT cells. The flow cytometric analysis confirmed that almost the totality of wt iNKT cells, as their human counterpart, expresses WASp (Figure 6).

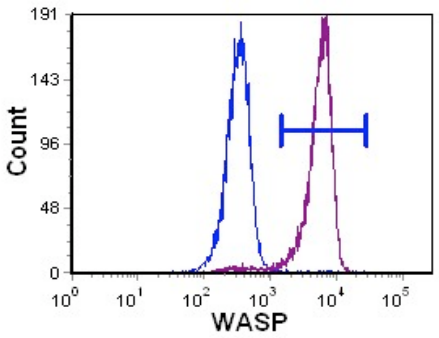


Figure 6: WASp expression by murine wt iNKT cells. Hepatic leukocytes from wt mice were stained with anti-B220, anti-CD3, anti-WASp Abs and with αGalCer loaded CD1d tetramers. The graph displays the flow cytometric analysis of WASp expression in a negative control (blue histogram) and in $B220^{-}CD3^{+}$CD1d tetramers^{+} wt iNKT cells (purple histogram).

5.2.2 Reduced iNKT cell frequency and number in *was*$^{-/-}$ mice

We next investigated the distribution of iNKT cells in the thymus, liver and spleen of was-/- mice by flow cytometric analysis. In mice iNKT cells can be easily detected by FACS using αGalCer loaded CD1d tetramers, which are specifically bound only by the semi-invariant TCR of iNKT cells. As displayed in Figure 7A and 7B, the percentage of iNKT cells specifically stained with αGalCer loaded CD1d tetramers was not significantly decreased in *was*$^{-/-}$ thymi. On the contrary, a lower iNKT cell proportion was found in the periphery of WASp deficient mice since iNKT cell percentages were 2 and 3 fold reduced in spleen and liver, respectively, of *was*$^{-/-}$ animals compared to wt controls. However, the absolute number of iNKT cells was significantly reduced in all three compartments of *was*$^{-/-}$ mice in comparison to wt mice (Figure 7C). Thus, we can conclude that the lack of WASp causes a mild decrease in the thymus together with a stronger reduction in the amount of iNKT cells in the periphery.

In order to understand whether WASp deficiency might affect the usage of certain TCR Vα and Vβ chains, altering in turn the frequency of other T cell population, we evaluated by FACS analysis the TCR Vβ repertoire and some families of TCRVα. The analysis was performed on CD3^{+} cells obtained from the spleens of 4 *was*$^{-/-}$ mice and C57 Bl6 controls (Figure 8A and 8B). From the data obtained, it emerged that the expression of TCR Vα or Vβ genes by *was*$^{-/-}$ and wt T cells was comparable, with the only exception of Vα2 that is significantly less expressed by was$^{-/-}$ T cells. Taken together these data demonstrate a consistent reduction in the pool of TCR^{+} iNKT cells as opposite to normal generation and usage of different TCR Vα and Vβ chains by *was*$^{-/-}$ T cells.

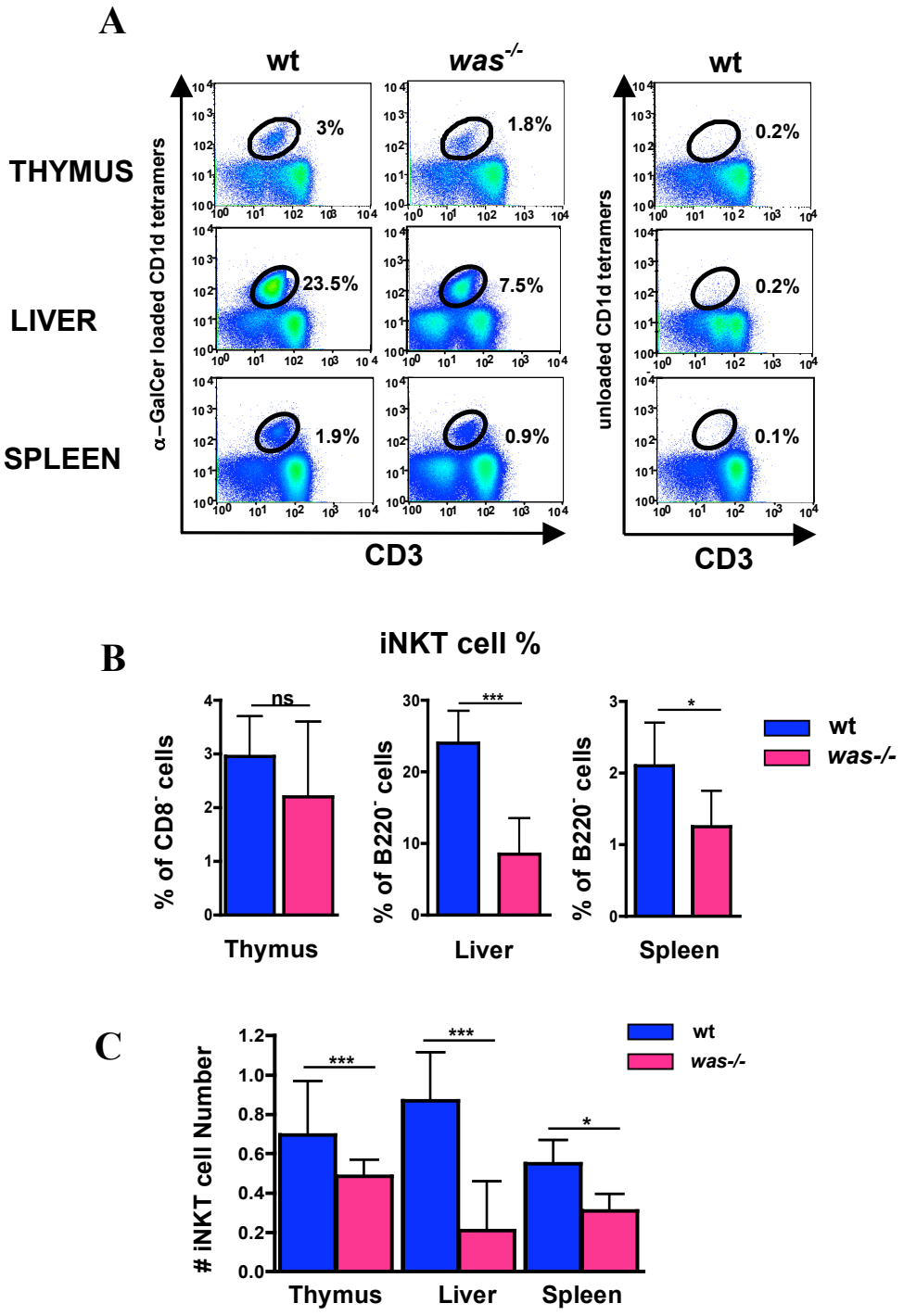


Figure 7. iNKT cell frequencies and number in *was*$^{-/-}$ mice. (A) iNKT cells were analyzed by FACS in thymus, liver and spleen of C57BL/6 wild type (wt) and *was*$^{-/-}$ mice. Thymocytes were stained with anti-CD8, anti-CD3 mAbs and CD1d tetramers (αGalCer loaded or unloaded) while hepatic leukocytes and splenocytes were stained with anti-B220, anti-CD3 mAbs and CD1d tetramers (αGalCer loaded or unloaded). After gating on CD8^{-} or B220^{-} cells, iNKT cells were identified as CD3^{+} CD1d tetramers^{+} cells. The percentage of iNKT cells is indicated in each plot. The data are representative of at least ten mice per group analyzed in three independent experiments, which are all summarized in (B) and in (C). (C) Absolute numbers of iNKT cells were determined multiplying their percentage for the absolute cell count within each sample. For (B) and (C), bars represent median and interquartile range. * = $p<0.05$, *** = $p<0.001$.

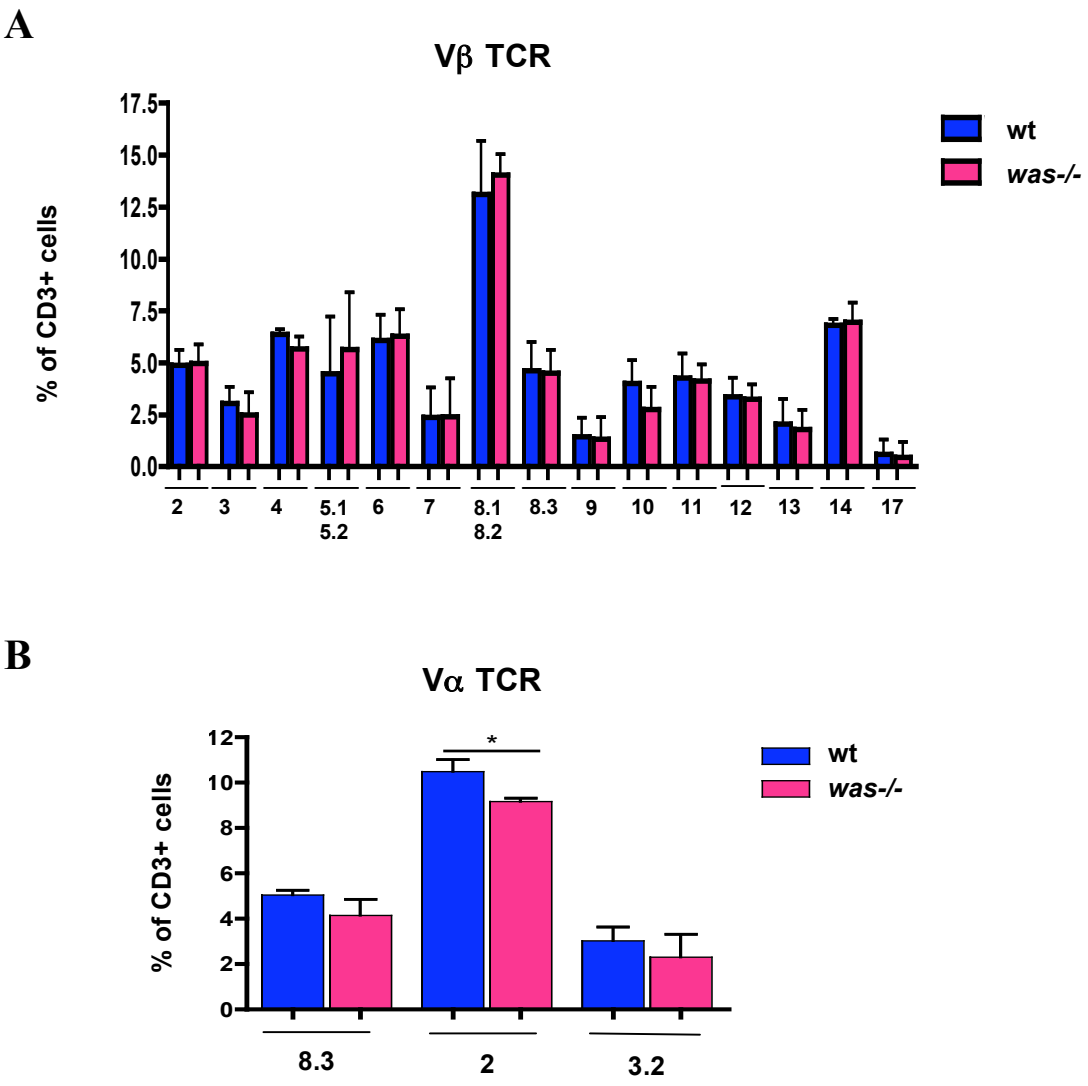


Figure 8. TCR Vβ and Vα expression in *was*$^{-/-}$ T lymphocytes. (A) TCR Vβ Repertoire was analyzed by FACS. Splenocytes from wt and *was*$^{-/-}$ mice were stained with anti-CD3 and with anti-TCR Vβ mAbs from TCR Vβ Screening Panel (BD). The percentages of the each different TCR Vβ^{+} CD3^{+} cells are depicted in the graph.(B) Splenocytes from wt and *was*$^{-/-}$ mice were stained with anti-CD3 and with anti-TCR Vα2, anti-TCR Vα3.2 and anti-TCR Vα8.3 mAbs. The percentages of the each different TCR Vα^{+} CD3^{+} cells are depicted in the graph. In (A) and (B) the data are representative of four mice per group and bars represent median and interquartile range. * = $p<0.05$.

5.2.3 Regular phenotype and subset distribution of *was*$^{-/-}$ iNKT cells

Based on CD4 expression, it is possible to distinguish two iNKT cell subsets: $CD4^+CD8^-$ and $CD4^-CD8^-$, respectively referred as $CD4^+$ and DN (Double Negative) iNKT cells. $CD4^+$ and DN iNKT cells are characterized by peculiar tissue distribution and cytokine profile (157, 158). We next investigated whether one of them is selectively altered in the absence of WASp. However, with the only exception of DN splenic iNKT cells, both $CD4^+$ and DN iNKT cells were found reduced in the thymus, liver and spleen of *was*$^{-/-}$ mice in comparison to the normal controls (Figure 9) and no skewing toward one particular subset was observed.

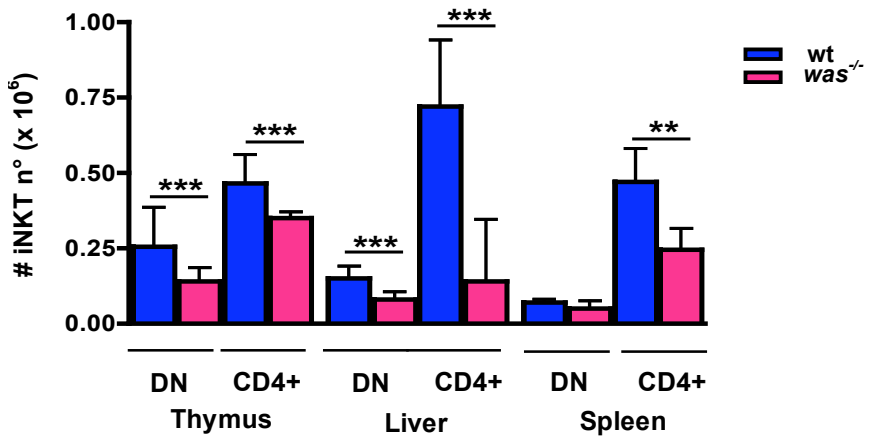


Figure 9. Analysis of iNKT cell subsets in WASp null mice. (A) iNKT cells were analyzed by flow cytometry as described in Figure legend 7 and further stained with anti-CD4 antibodies. Comparison of the absolute number of $CD4^+$ or $CD4^-$ (DN) iNKT cells in the Thymus, Liver and Spleen in wt versus *was*$^{-/-}$ mice is shown. Bars represent median and interquartile range of eight mice per group. ** = P<0,005, *** = P<0.001

Other subtypes of murine iNKT cells, with different avidity for CD1d-glycolipid complexes, can be identified on the basis of TCR Vβ chain usage (205, 206). Indeed, the majority of iNKT cells expresses a Vα14-Jα18 TCRα chain, which pairs preferentially with either Vβ8 or Vβ7 TCRβ chains. As shown in Fig 10A, we found a normal frequency of thymic TCR Vβ7 positive *was*$^{-/-}$ iNKT cells. In spite of that, we observed a small but significant reduction of *was*$^{-/-}$ iNKT cells bearing TCR Vβ8 chain. Moreover TCR Vβ8 positive, but not TCR Vβ7 positive *was*$^{-/-}$ iNKT cells, displayed a lower surface expression level of the respective TCR Vβ chain in comparison to wt iNKT cells, as suggested by the analysis of MFI. Altogether these data demonstrate that the lack of WASp causes a significant overall decrease in the iNKT cell population.

An important hallmark of murine iNKT cells is the surface expression of the activation markers (149). To assess whether WASp is involved in the acquisition of this mature phenotype we analyzed CD25, CD44 and CD69 expression on wt and *was*$^{-/-}$ iNKT cells. Our analysis revealed a frequency of CD25^{+}, CD44^{+} and CD69^{+} *was*$^{-/-}$ iNKT cells similar to that of the wt counterpart (Fig 10B). Nonetheless the lack of WASp causes a significant decrease in the expression level of the CD44 molecule, both in thymus and in the periphery.

Overall these results demonstrate a normal activated phenotype of *was*$^{-/-}$ iNKT cells.

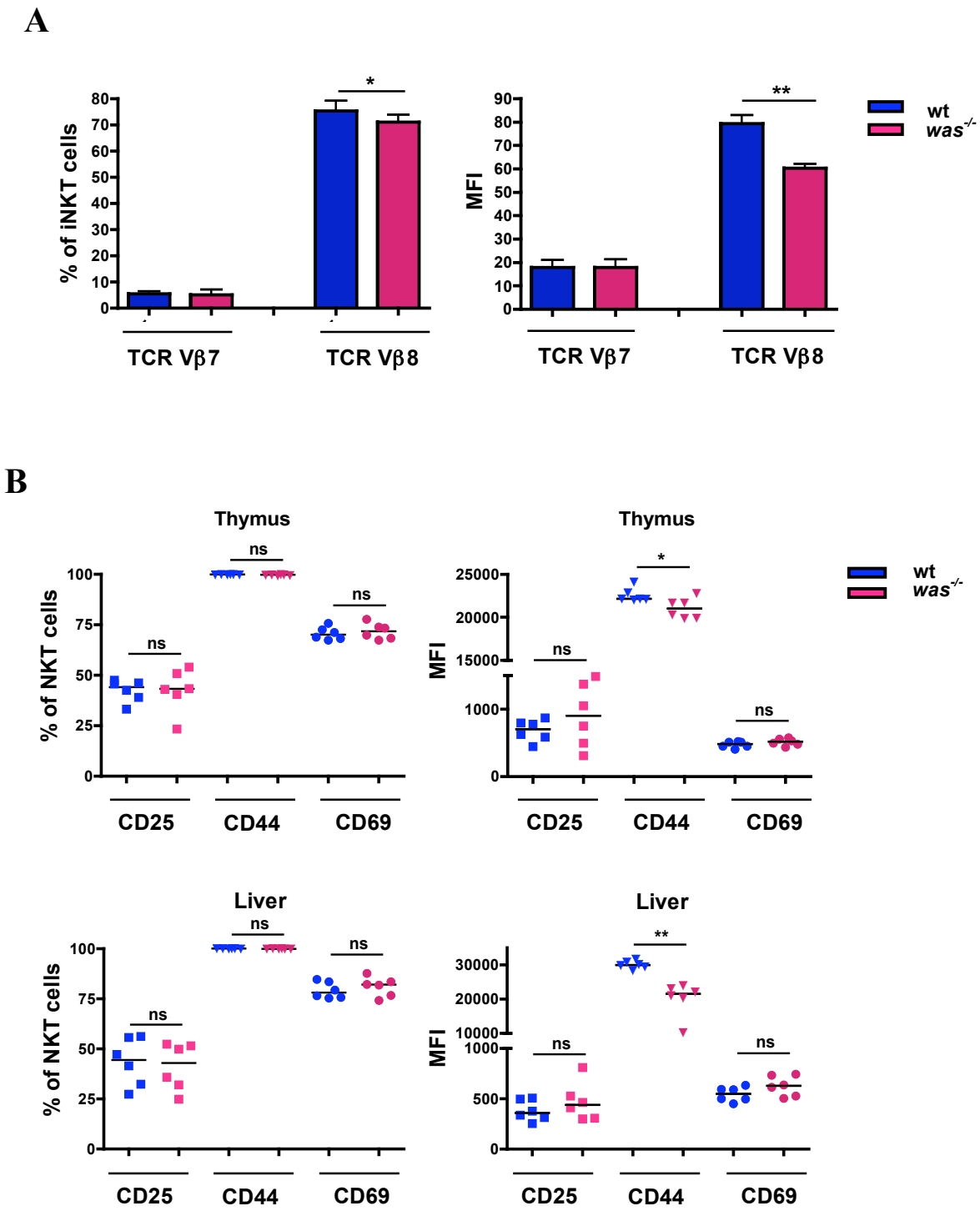


Figure 10. TCR Vβ and Activation Markers expression in *was*$^{-/-}$ iNKT cells. (A) Thymic iNKT cells from wt and *was*$^{-/-}$ mice were analyzed by flow cytometry as described in Figure legend 7 and further stained with anti-TCR Vβ7 or Vβ8 mAbs. The graph shows the percentages and Mean Fluorescence Intensity (MFI) of TCR Vβ7 and Vβ8 positive iNKT (CD8^{-}CD3^{+}CD1d tetramers^{+}) cells. Bars represent median and interquartile range of six mice per group.
(B) Thymic and hepatic iNKT cells were also stained with anti-CD25, anti-CD44 and with anti-CD69 mAbs. The percentages and MFI of CD25^{+}, CD44^{+} and CD69^{+} wt and *was*$^{-/-}$ iNKT cells are depicted in the graph. Data are representative of six mice per group and bars represent median value of each group. * = p<0.05 and ** = P<0,005.

5.2.4 Developmental block of *was*$^{-/-}$ iNKT cells

In order to understand whether the decreased iNKT cell frequency and number of iNKT cells in *was*$^{-/-}$ mice was caused by an altered thymic development, we first analyzed the expression of CD1d on DP thymocytes, whose expression is required for iNKT cell positive selection (140). As displayed in Fig.11, DP thymocytes from *was*$^{-/-}$ mice express CD1d molecules identically to wt control.

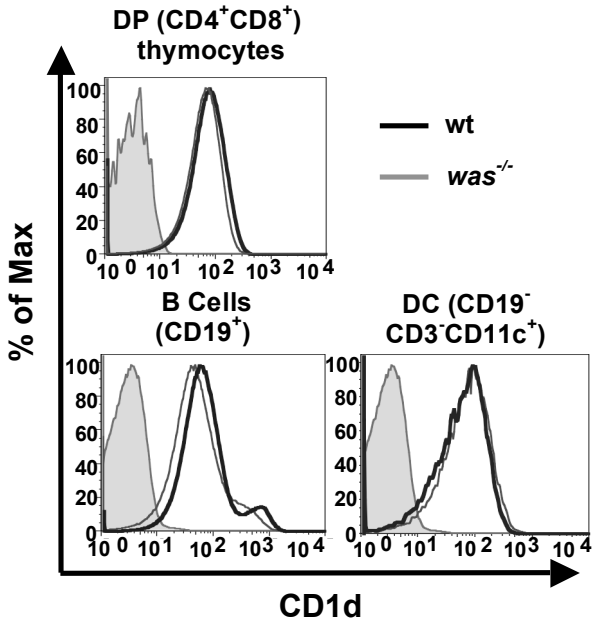


Figure 11. CD1d expression in *was*$^{-/-}$ mice. CD1d expression was analyzed in thymic $CD4^+CD8^+$ lymphocytes (upper) and in splenic $CD19^+$ B cells and $CD19^-$, $CD3^-$, $CD11c^+$ DC in wt (thick line) and *was*$^{-/-}$ mice (thin line). Data are representative of six mice per group.

We next investigated the maturation steps of iNKT cells following positive selection, using CD44 and NK1.1 markers (152). As shown in Fig. 12A, *was*$^{-/-}$ iNKT cells were mainly arrested at CD44^{+} NK1.1^{-} stage and only a minor fraction became NK1.1^{+} both in the thymus and in periphery. The analysis of the absolute number of iNKT cells during the different maturation steps confirmed that WASp does not affect the earliest developmental phase, where *was*$^{-/-}$ iNKT cells are normally present (Fig. 12B). Conversely *was*$^{-/-}$ iNKT cells accumulate at stage 2 (CD44^{+} NK1.1^{-}) without progressing to stage 3 (CD44^{+} NK1.1^{+}), suggesting a potential role of WASp in regulating the late phases of the differentiation process.

In the thymus, CD44^{+} NK1.1^{-} iNKT cells can upregulate another NK cell marker, the DX5 molecule (152). DX5 expression defines one more developmental phase occurring before NK1.1 upregulation. However *was*$^{-/-}$ iNKT cells were not defective in DX5 upregulation (Fig. 12C) indicating that WASp requirement is crucial only in the final stage of iNKT cell development.

Different events can contribute to the final maturation of iNKT cells in the periphery. Among these a very important role is played by CD1d recognition, since NK1.1^{-} iNKT cells fail to properly complete their maturation in the absence of CD1d (209). To address this point, we examined CD1d expression in the periphery of *was*$^{-/-}$ mice, finding expression levels comparable to that of the wt (Fig. 11). These data rule out the possibility that an altered CD1d expression in the periphery may have a role in developmental block of *was*$^{-/-}$ iNKT cells.

As next we wanted to assess whether the developmental defect of iNKT cells in *was*$^{-/-}$ mice is cell autonomous. To unravel this issue we generated mixed BM chimeras by reconstituting irradiated CD45.1 mice with a mixture of equal number of CD45.2 lineage negative (lin^{-}) cells obtained from BM of *was*$^{-/-}$ and wt mice. In this model, CD45.2 *was*$^{-/-}$ iNKT cell precursors develop in the presence of donor-derived CD45.2 DP thymocytes and hematopoietic cells from either *was*$^{-/-}$ or wt mice. In case of an iNKT cell intrinsic defect, the presence of wt cells would not be sufficient to rescue the development of *was*$^{-/-}$ iNKT cells. BM chimeras were analyzed 7-9 weeks post transplantation and evaluated for the expansion and maturation of iNKT cells derived from either *was*$^{-/-}$ or wt hematopoietic precursors. As shown in Fig. 13, the percentage of iNKT cells developing in *was*$^{-/-}$ mice reconstituted with *was*$^{-/-}$ lin^{-} cells (ko→ko control) was reduced both in the thymus and more markedly in the periphery in

comparison to that observed in wt recipients transplanted with wt precursors (wt→wt control). Both mixed BM chimeras generated in *was*$^{-/-}$ and wt recipients displayed a level of iNKT cells very similar to wt→wt control, suggesting the same capacity of wt and was$^{-/-}$ recipients to support iNKT cell generation. The analysis of maturation of donor iNKT cells based on NK1.1 expression confirmed the complete developmental block of *was*$^{-/-}$ iNKT cells in ko→ko controls as opposed to successful maturation of WASp^{+} iNKT cells in wt→wt control (Fig.13B). In the BM chimeras, whereas WASp^{+} iNKT cells could acquire the mature phenotype in both recipients, WASp^{-} iNKT cells were unable to upregulate NK1.1 even in the presence of hematopoietic cells derived from wt progenitors.

Altogether, these findings demonstrate that the lack of WASp determines an iNKT cell autonomous defect that impairs the maturation, survival and/or expansion of these cells.

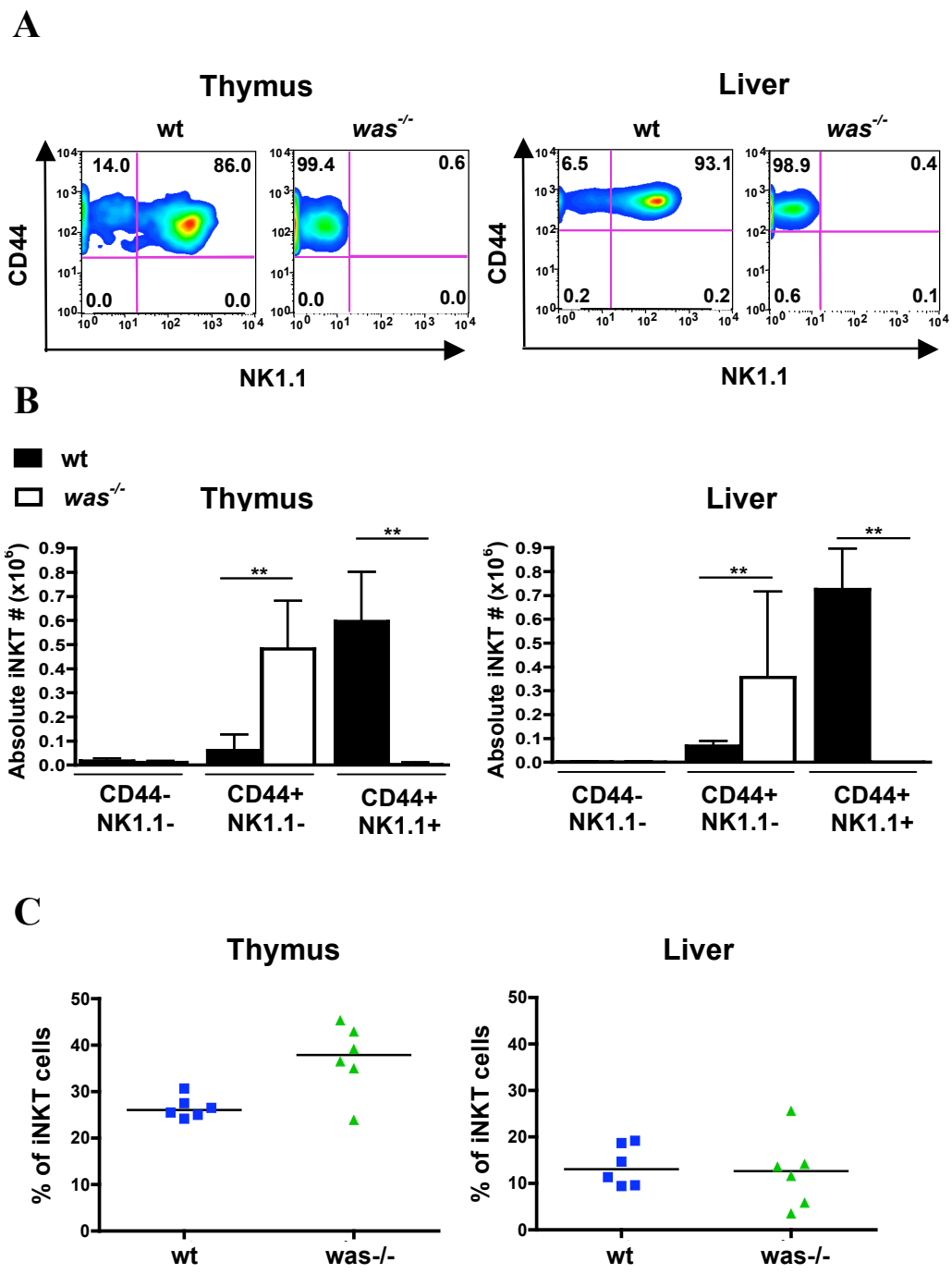


Figure 12. iNKT cell maturation in the absence of WASp. (A) Thymocytes and hepatic leukocytes from wt and *was*$^{-/-}$ mice were stained with anti-CD8 or anti-B220, anti-CD3, anti-CD44, anti-NK1.1 mAbs and αGalCer loaded CD1d tetramers. Maturation of iNKT cells (CD1d tetramer^{+}, CD3^{+}, CD8^{-}) was assessed based on the expression of CD44 and NK1.1.Data are representative of six mice per group analyzed in two independent experiments. (B) Absolute numbers of iNKT cells (CD1d tetramer^{+}, CD3^{+}, CD8^{-}) in thymus and liver of wt and *was*$^{-/-}$ mice. Bars represent median and interquartile range of six mice per group. ** = $p<0.005$. (C) DX5 expression by iNKT cells (CD1d tetramer^{+}, CD3^{+}, CD8^{-}/B220^{-}). Data are representative of six mice per group analyzed in two independent experiments. Bars represent median value of each group.

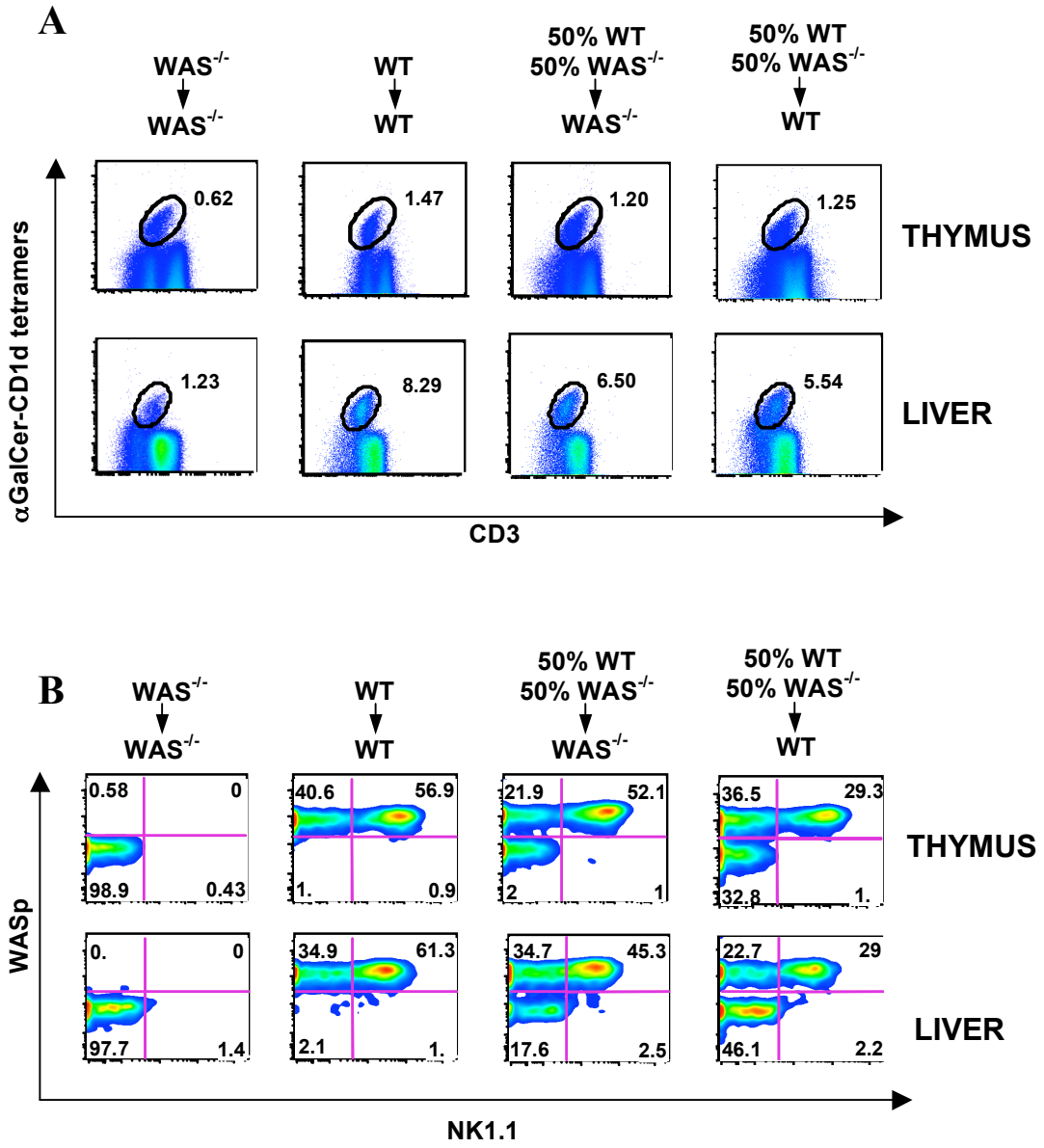


Figure 13. Generation of Mixed Bone Marrow Chimeras. (A) iNKT cells were analyzed by flow cytometry in thymus and liver of wt or *was*$^{-/-}$ recipient mice (CD45.1) transplanted with *was*$^{-/-}$ lin^{-} cells, wt lin^{-} cells or with a mixture of 50% wt and 50% *was*$^{-/-}$ lin^{-} obtained from CD45.2 mice. Thymocytes and hepatic leukocytes were surface stained with αGalCer loaded CD1d tetramers, with anti-CD3 and anti-CD8 (thymocytes) or anti-B220 (hepatic leukocytes) mAbs. The percentage of iNKT cells (CD8^{-} or B220^{-} CD3^{+}CD1d tetramers^{+} cells) is indicated in each plot. (B) Maturation of iNKT cells in thymus and liver of BM chimera mice. After gating on donor CD45.2^{+} iNKT cells were further analyzed for NK1.1 and WASp expression. The percentages of mature (NK1.1^{+}) and immature (NK1.1^{-}) iNKT cells from wt donors (WASp^{+}) or *was*$^{-/-}$ donors (WASp^{-}) are indicated in each plot. Data are representative of at least three mice per group from a single experiment.

5.2.5 Evaluation of apoptotic cell death and homeostatic proliferation in murine iNKT cells

A possible explanation for the reduced pool of mature iNKT cells in *was*$^{-/-}$ mice could be the increase of apoptotic cell death and/or the impaired homeostatic proliferation of iNKT cells lacking WASp.

Among the different ways to measure apoptosis one efficient approach is to evaluate the activity of caspases, key mediators of the programmed cell death process. To this aim, thymocytes and hepatic leukocytes from wt and *was*$^{-/-}$ mice were incubated with CaspACE, a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) able to bind to activated caspases. Our monitoring of CaspACE positive cells by fluorescence detection did not reveal any remarkable increment of caspase activity in *was*$^{-/-}$ iNKT cells (Fig.14A). Even the comparison between immature NK1.1^{-} iNKT cells from wt and *was*$^{-/-}$ mice did not show any significant difference in activated caspase levels (Fig. 14B).

To find out if a reduced homeostatic proliferation of iNKT cells accounts for the lower iNKT cell number of *was*$^{-/-}$ mice, we evaluated the *in vivo* turnover of iNKT cells. To this aim we adopted a labeling method based on the usage of Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU), a synthetic nucleoside analogue of thymidine. Indeed as previously published following *in vivo* administration of BrdU for 6 days (232) many thymic and peripheral iNKT cells normally proliferate, incorporating BrdU. As expected a detectable wt iNKT cell fraction was BrdU^{+} at day 6 (Fig. 15A). Of note, even if normally proliferating in the periphery, *was*$^{-/-}$ iNKT cells presented a significantly higher turnover in the thymus. Faster BrdU labeling of thymic iNKT cells most likely reflected the increased proportion of immature iNKT cells in *was*$^{-/-}$ mice, as NK1.1^{-} iNKT cells were demonstrated to be the iNKT cell fraction most actively proliferating (164). Nevertheless when the proliferation analysis was restricted to the NK1.1^{-} iNKT cell fraction it disclosed homeostatic proliferation ability severely compromised in cells lacking WASp (Fig. 15B).

Altogether these results indicated the impaired proliferation capacity of *was*$^{-/-}$ iNKT cells as the responsible for reduced iNKT cell pool in *was*$^{-/-}$ mice.

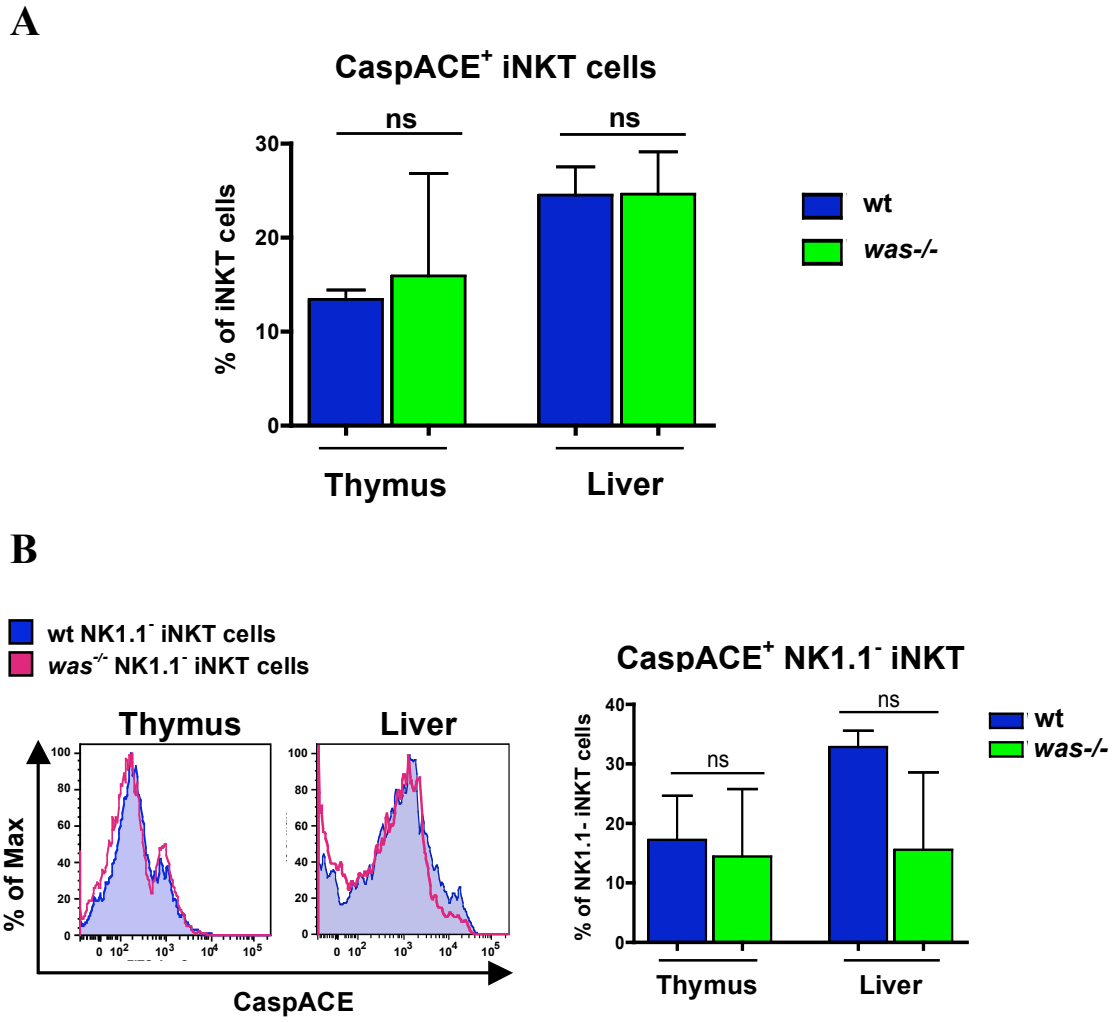


Figure 14. Activated caspase expression in *was*$^{-/-}$ iNKT cells. (A) Flow cytometric analysis of activated caspase levels by iNKT cells. Thymocytes and hepatic leukocytes from wt and *was*$^{-/-}$ mice were stained with CaspACE, αGalCer loaded CD1d tetramers, with anti-CD3 and anti-CD8 (thymocytes) or anti-B220 (hepatic leukocytes) mAbs. The graph shows percentages of CaspACE^{+} iNKT cells (CD8^{-} or B220^{-} CD3^{+}CD1d tetramers^{+} cells) from 7 mice. (B) Evaluation of activated caspase levels by NK1.1^{-} iNKT cells. After gating on iNKT population, the cells were further analyzed for NK1.1 and CaspACE expression. On the left a representative CaspACE expression by NK1.1^{-} iNKT cells from wt (blue) and *was*$^{-/-}$ (purple) mice In the graph (on the right) the percentages of CaspACE^{+} NK1.1^{-} iNKT cells from 7 mice are depicted. Bars represent the median value and the interquartile range.

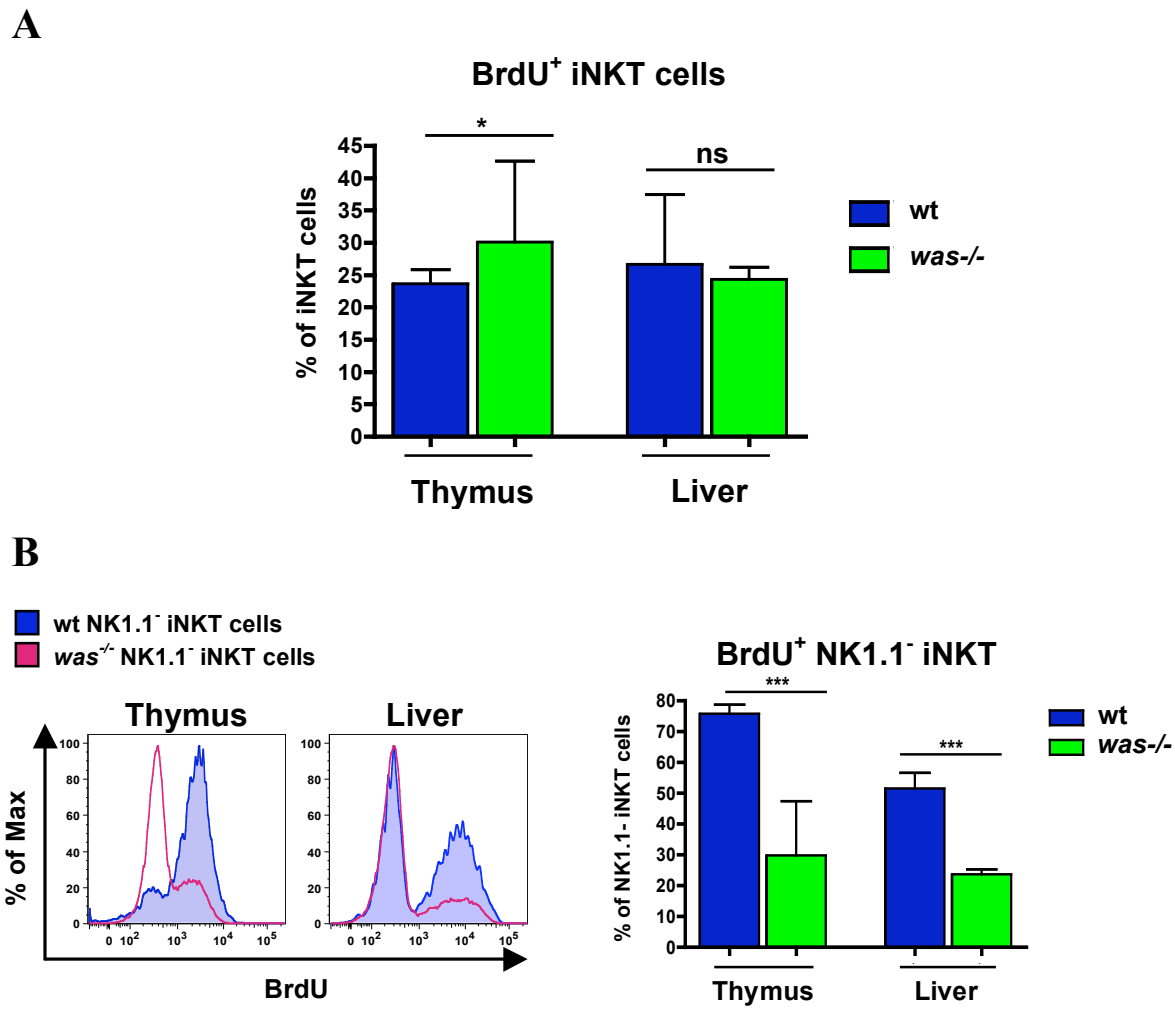


Figure 15. Homeostatic iNKT cells proliferation in *was*$^{-/-}$ mice. (A) Flow cytometric analysis of BrdU incorporation by iNKT cells. BrdU was injected intraperitoneally and given in drinking water daily. Mice were analyzed at day 6. The graph shows percentages of BrdU^{+} iNKT cells (CD8^{-} or B220^{-} CD3^{+}CD1d tetramers^{+} cells) from thymus and liver of 7 mice. (B) Evaluation of BrdU incorporation by NK1.1^{-} iNKT cells. On the left a representative BrdU uptake by NK1.1^{-} iNKT cells from wt (blue) and *was*$^{-/-}$ (purple) mice. In the graph (on the right) the percentages of BrdU^{+} NK1.1^{-} iNKT cells from 7 mice are depicted. Bars represent the median value and the interquartile range.

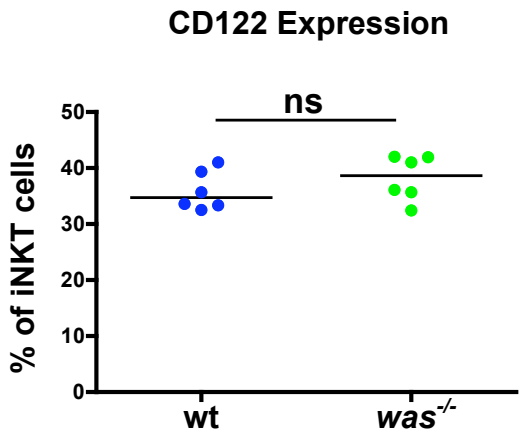
5.2.6 Proliferation of *was*$^{-/-}$ iNKT cells in response to IL-15

Among the requirement for iNKT cell expansion and/or survival, a crucial role seems to be played by the cytokine IL-15. Indeed, impaired IL-15 receptor signaling results into significant reduction of thymic and peripheral iNKT cell numbers (232). Therefore we first measured on wt and *was*$^{-/-}$ iNKT cells the expression of IL-2Rβ chain (CD122), which is a component of the signaling complex for IL-15. As shown in Figure 16A, the absence of WASp did not alter the ability to express CD122 on iNKT cells.

Moreover we analyzed the proliferative response of *was*$^{-/-}$ iNKT cells to IL-15. To this aim, CD8 depleted thymocytes from wt and *was*$^{-/-}$ mice were labeled with CSFE and put in culture with human recombinant IL-15 (Fig.16B). After 4 days of culture in the presence of IL-15, almost the totality of *was*$^{-/-}$ iNKT cells, as well as wt controls, underwent to cellular proliferation, as demonstrated by the similar dilution of CSFE.

Overall these data ruled out a deficient IL-15 signaling as the responsible of the reduced iNKT cell pool in *was*$^{-/-}$ mice.

A



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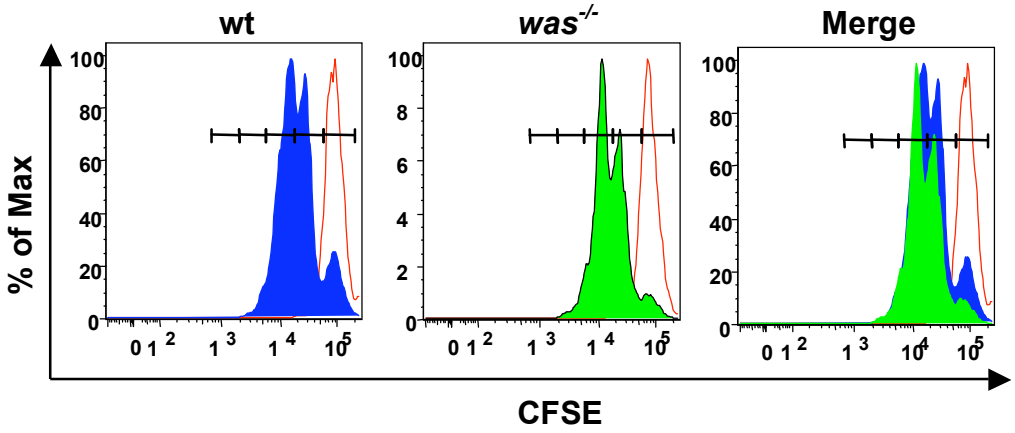

Figure 16. IL-15 receptor receptor signaling in *was*$^{-/-}$ mice. (A) Flow cytometric analysis of IL-2Rβ chain (CD122) expression by thymic iNKT cells. The graph summarizes data from two independent experiments. Bar represents the median value of each group. (B) *In vitro* iNKT cell proliferation in response to IL-15. CD8 depleted thymocytes from wt and *was*$^{-/-}$ mice were labeled with CFSE and put in culture for 4 days with 100 ng/ml human recombinant IL-15. The graph shows CFSE dilution of wt (blue) and *was*$^{-/-}$ (green) iNKT cells. The red empty histogram represents CFSE dilution of cells put in culture without IL-15. Data are representative of three independent experiments.

5.3 FUNCTIONAL CHARACTERIZATION OF MURINE *was*$^{-/-}$ iNKT CELLS

5.3.1 Reduced *in vivo* cytokine production upon αGalCer injection in *was*$^{-/-}$ mice

To determine whether WASp plays a role also in the effector function of iNKT cells we evaluated the *in vivo* response to αGalCer, a synthetic glycosphingolipid that elicits a prompt and selective activation of iNKT cells through the CD1d molecule. Indeed after αGalCer stimulation, iNKT cells produce huge amounts of Th1 and Th2 cytokines. We injected αGalCer into *was*$^{-/-}$ mice and wt controls and measured the cytokine production in the sera of treated animals 3, 6, 12 and 24 hours from the stimulation. As expected, the *in vivo* activation of wt iNKT cells resulted in consistent production of the prototypical IL-4 and IFN-γ cytokines. Although in *was*$^{-/-}$ mice the kinetics of cytokine production was similar to that of wt mice, the amount of IL-4 and IFN-γ in *was*$^{-/-}$ sera was significantly reduced at every time-point tested (Fig.17A). Similar results were also found for other cytokines produced by activated iNKT cells, as the growth factor IL-2 and GM-CSF, which were consistently reduced in *was*$^{-/-}$ mice at the time of maximum production (Fig.17B). Furthermore we detected a drop in the levels of IL-10, TNFα and IL-6 in *was*$^{-/-}$ sera in comparison to wt at the earliest time point following αGalCer stimulation. Collectively these results demonstrate a profound decrease in the amount of different cytokines produced upon αGalCer injection in *was*$^{-/-}$ mice.

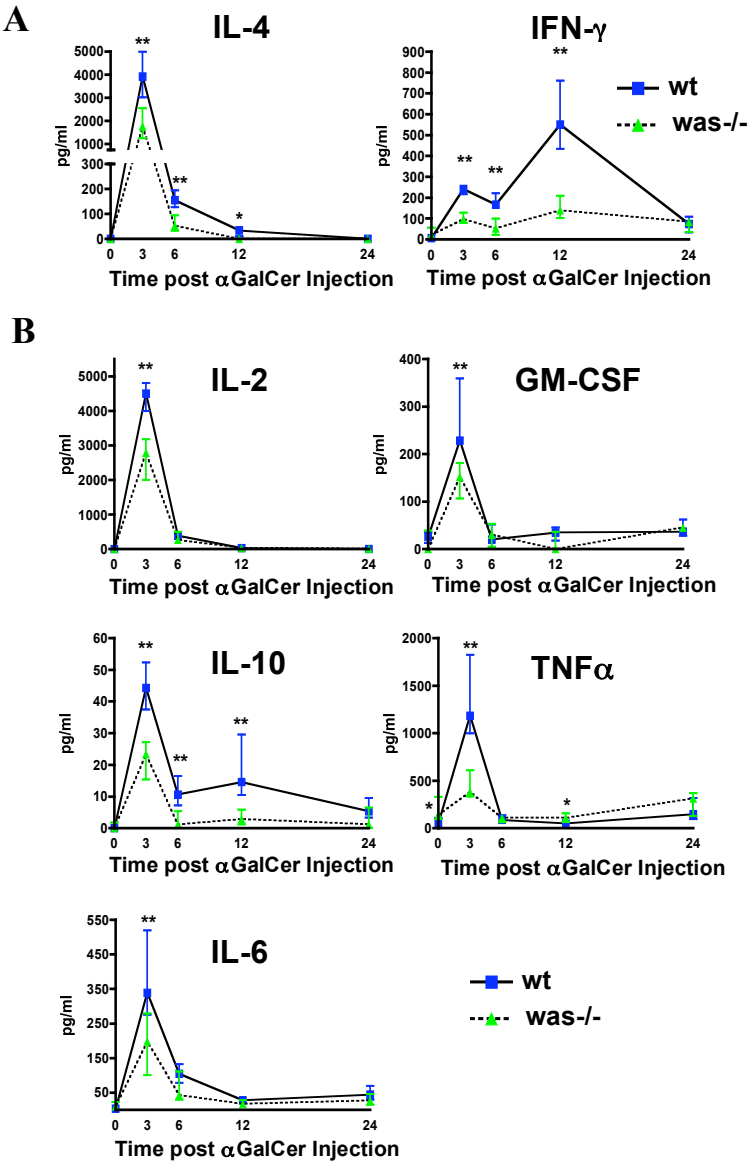

Figure 17. Cytokine production by *was*$^{-/-}$ mice upon αGalCer injection. (A) *In vivo* IL-4 and IFN-γ production upon αGalCer administration in wt and *was*$^{-/-}$ mice. Sera were analyzed by Bioplex Technology at 3, 6, 12 and 24 hours upon injection. (B) At 3, 6, 12 and 24 hours upon αGalCer injection it was also measured the level of IL-2, GM-CSF, IL-10, TNFa and IL-6 in the sera of treated animals. In (A) and in (B) the graphs show the amount of cytokines produced by 6 wt and 6 *was*$^{-/-}$ mice. Blue square and green triangle represent respectively the median value of wt and *was*$^{-/-}$ mice group. The vertical bars represent interquartile range of each group. * = $p<0.05$ and ** = $p<0.005$.

5.3.2 Functional impairment of *in vivo* activated *was*$^{-/-}$ iNKT cells

The lower cytokine levels described above could be caused by the reduced iNKT cell number of *was*$^{-/-}$ mice, but also by the impaired ability of *was*$^{-/-}$ iNKT cells to produce cytokines. To address this issue, we administered αGalCer to *was*$^{-/-}$ or wt mice to *in vivo* activate iNKT cells and we analyzed *ex vivo* the intracellular level of the most representative cytokines produced by iNKT cells. Forty-five minutes after αGalCer injection, a significant fraction of wt iNKT cells produced considerable amount of intracellular IL-4 and IFN-γ, whereas *was*$^{-/-}$ iNKT cells showed an impaired production of both cytokines (Fig. 18).

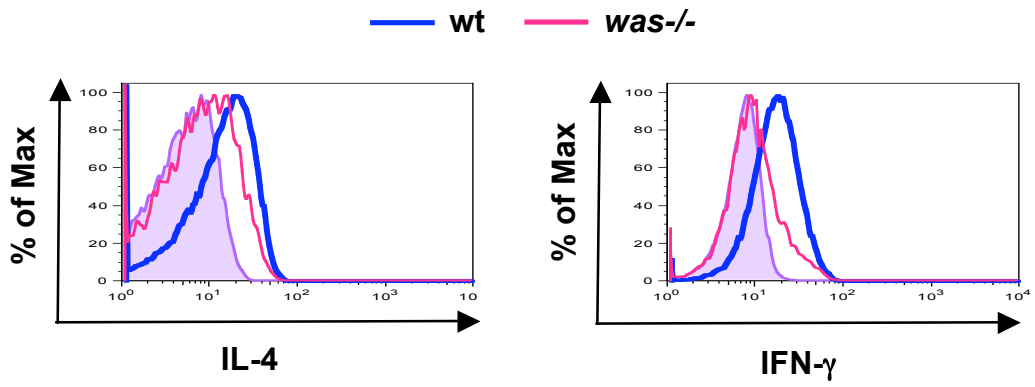

Figure 18. Cytokine production by *was*$^{-/-}$ iNKT cells upon *in vivo* stimulation. IL-4 and IFN-γ production were measured in wt and *was*$^{-/-}$ mice at the single iNKT cell level. wt and *was*$^{-/-}$ mice were injected with αGalCer and after 45 minutes hepatic leukocytes were isolated and stained with αGalCer loaded CD1d tetramers, anti-CD3, anti-IL-4 and anti-IFN-γ mAbs. Representative analysis of IL-4 and IFN-γ intracellular production by iNKT cells (CD3^{+}, CD1d tetramers^{+}) from wt (blue line) and *was*$^{-/-}$ (purple line) mice. Violet histograms represent IL-4 or IFN-γ production by untreated wt mice. Data are from one experiment representative of three.

Another measure of iNKT cell functional ability is given by their capacity to *in vivo* expand following αGalCer stimulation (295). To check this ability, wt and *was*$^{-/-}$ mice were treated with αGalCer and iNKT cell expansion was evaluated by flow cytometry at day 3, 7 and 11 after the injection. The analysis of iNKT cell percentage and absolute number at the different time-points revealed, as expected, a peak of expansion of wt iNKT cell at day 3 followed by a contraction at day 7. Conversely, *was*$^{-/-}$ iNKT cells showed a delayed kinetics, with the maximum expansion at day 7, followed by a contraction at day 11 (Fig.19A). Of note the absence of WASp seems to cause a delay likewise in the surface upregulation of TCR upon iNKT cell stimulation, since the percentage of *was*$^{-/-}$ iNKT cells at day 3 is significantly decreased in comparison to that at day 0.

The increase in absolute numbers of *was*$^{-/-}$ iNKT cells induced by the αGalCer was significant compared to the untreated mice, however, the capacity of *was*$^{-/-}$ iNKT cells to expand was substantially lower than that of wt iNKT cells, as shown by the differences in the fold increase values (Fig.19B).

Altogether, these results provide proof of evidence that *in vivo* activated *was*$^{-/-}$ iNKT cells are not properly functional.

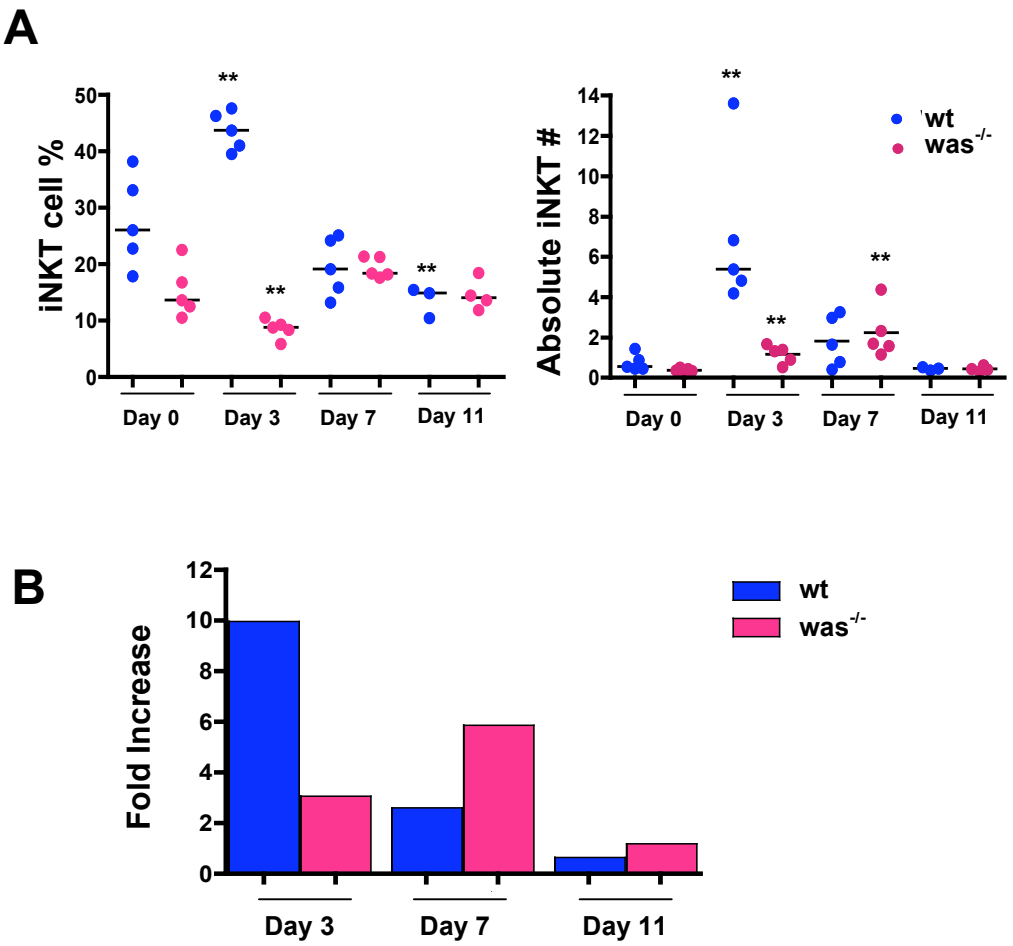

Figure 19. ***was*$^{-/-}$ iNKT cell *in vivo* expansion upon αGalCer stimulation.** (A) wt and *was*$^{-/-}$ mice were injected with αGalCer and iNKT cell *in vivo* expansion was measured by flow cytometry at 3, 7 and 11 days following the injection. To evaluate iNKT cell expansion hepatic leukocytes were stained with anti-B220, anti-CD3 mAbs and with αGalCer loaded CD1d tetramers. iNKT cell percentages and absolute numbers of iNKT cells were determined as described in Figure Legend 7. The graph shows iNKT cell number of 5 wt and 5 *was*$^{-/-}$ mice/group with the exception of day 11 (3 wt and 4 *was*$^{-/-}$ mice). Statistic significance is referred to the difference between each group and its relative untreated control. ** = $p<0.005$ of each group compared to untreated mice (Day 0) (B) Fold increase of iNKT cell number at day 3, day 7 and day 11 was calculated as ratio of, respectively, the mean value of iNKT cell number at day 3, day 7 or day 11 and the mean value of iNKT cell number from untreated mice.

5.3.3 Reduced help to antigen specific humoral responses by *in vivo* activated *was*$^{-/-}$ iNKT cells

We next checked the *in vivo* ability of *was*$^{-/-}$ iNKT cells to support antigen specific T cell dependent antibody responses. It was indeed demonstrated that the activation of iNKT cells at the moment of immunization with protein antigens results in an enhanced secretion of antigen specific immunoglobulin (253).

To this aim wt and *was*$^{-/-}$ mice were immunized with the protein antigen TNP-KLH alone or admixed with αGalCer and after two weeks mice were boosted using the same vaccine formulation. Serum antibody levels were then evaluated two weeks following the second immunization (2wp2). As shown in Fig.20, the *in vivo* activation of iNKT cells caused, as expected, a significant increase of TNP-KLH specific IgG in wt mice. On the contrary activated *was*$^{-/-}$ iNKT cells were not able to support a relevant augmentation of specific IgG titer. Nonetheless other adjuvant as LPS significantly enhanced specific humoral responses in both wt and *was*$^{-/-}$ mice.

These results further confirm the *in vivo* functional deficiency of *was*$^{-/-}$ iNKT cells.

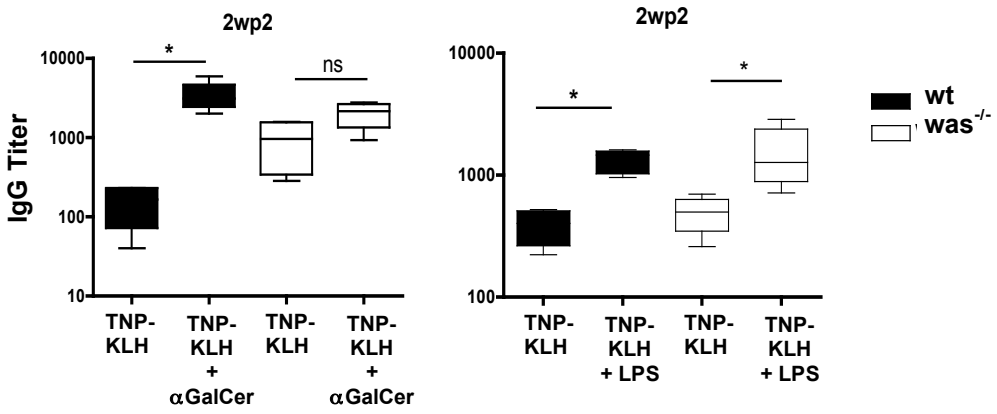

Figure 20. *In vivo* help of activated *was*$^{-/-}$ iNKT cell to specific B cell responses. wt and *was*$^{-/-}$ mice were immunized twice with TNP-KLH alone, with αGalCer or with LPS. The figure shows the antigen specific IgG titer at two weeks post second immunization (2wp2). Results are from one experiment representative of two, in which 4-5 mice were tested. ns = $p>0.05$, * = $p<0.05$.

5.3.4 Contribution of *was*$^{-/-}$ DCs to *was*$^{-/-}$ iNKT cell functional defect

The functional impairment of *was*$^{-/-}$ iNKT cells upon *in vivo* activation may be caused by a cell autonomous defect and/or by alterations in antigen presentation. In fact iNKT cells require, to be activated, the interaction of TCR with an antigen loaded CD1d molecule on the surface of the APC.

It seems possible that a reduced antigen presentation ability of *was*$^{-/-}$ APCs may contribute to the *in vivo* reduced functionality of *was*$^{-/-}$ iNKT cells, since *was*$^{-/-}$ DCs have a reduced ability to migrate, assemble podosomes and process particulate antigens (109).

To dissect the role of *was*$^{-/-}$ DCs in *was*$^{-/-}$ iNKT cell functional defect we performed an *in vitro* functional assay by putting in culture wt or *was*$^{-/-}$ αGalCer-pulsed DCs together with wt or *was*$^{-/-}$ iNKT cells in all possible combinations.

After 48 hours of co-culture the amount of IL-4 and IFN-γ produced by wt iNKT cells activated by wt DCs were significantly higher than that released by *was*$^{-/-}$ iNKT cells activated by *was*$^{-/-}$ DCs, thus reproducing *in vitro* what we previously found *in vivo* (Fig. 21). *was*$^{-/-}$ DCs were not relevantly defective in driving IL-4 and IFN-γ production by wt iNKT cells, suggesting that *was*$^{-/-}$ DCs are competent in antigen presentation to iNKT cells. Anyway *was*$^{-/-}$ iNKT cells produced more abundant levels of IFN-γ, but not of IL-4, when they were activated by wt DCs rather than by *was*$^{-/-}$ DCs, indicating a partial contribution of *was*$^{-/-}$ DCs to the defective IFN-γ production by *was*$^{-/-}$ iNKT cells.

On the other hand when *was*$^{-/-}$ iNKT cells were activated by wt DCs, they secreted IFN-γ at the same level of wt iNKT cells but failed to produce normal amount of IL-4.

Overall these results indicated an intrinsic functional defect of *was*$^{-/-}$ iNKT cells in the pathway leading to IL-4 production. In contrast the contribution of an altered crosstalk between *was*$^{-/-}$ DCs and *was*$^{-/-}$ iNKT cells might account for the reduced IFN-γ production found upon *in vivo* activation of *was*$^{-/-}$ iNKT cells.

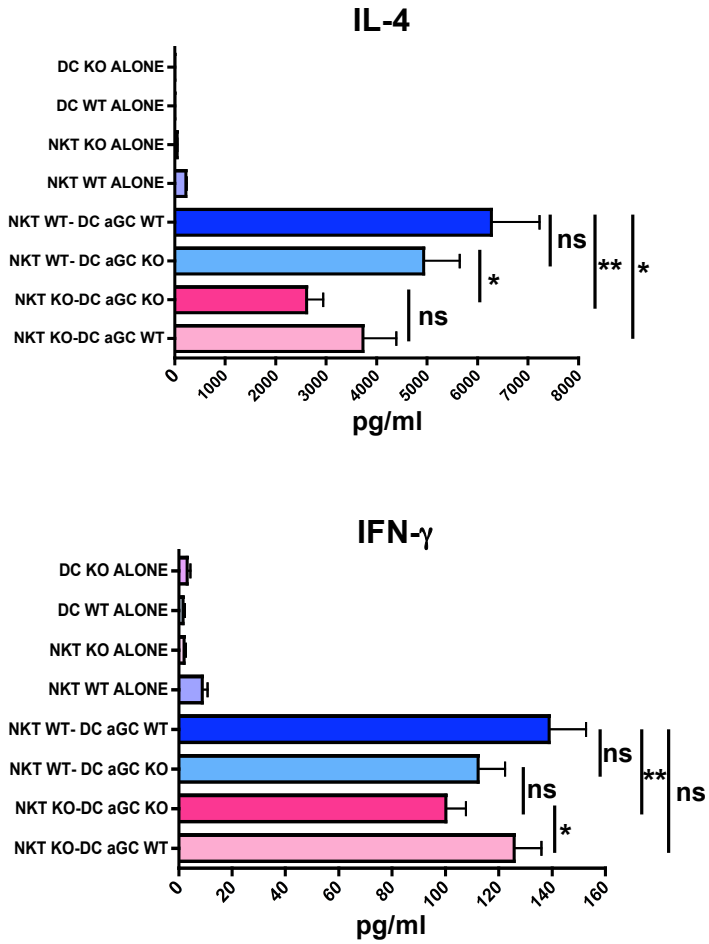

Figure 21. ***In vitro*** **activation of wt or *was*$^{-/-}$ iNKT cells by αGalCer loaded wt or *was*$^{-/-}$ DCs.** DCs from spleen of wt and *was*$^{-/-}$ mice were purified and pulsed overnight with αGalCer 100 ng/ml. αGalCer pulsed wt or *was*$^{-/-}$ DCs were put in culture with FACS sorted splenic wt or *was*$^{-/-}$ iNKT cells in 1:1 ratio. After 48hours of co-culture supernatants were collected for cytokine measurement by Bioplex technology. The graph shows the results from three independent experiments and for each experiment cells from at least 5 mice/group were pooled. Bars are mean and SEM for each group. *=$p<0.05$; ** = $p<0.005$

5.3.5 Normal ability of *was*$^{-/-}$ DCs to present αGalCer

Taking advantage of a recently developed antibody specific for the complex formed by the binding of αGalCer to murine CD1d (L363), the kinetics of αGalCer has been described (191). This study clearly shows that αGalCer is internalized in the endosomal compartment and later on presented by CD1d on APC surface. Because *was*$^{-/-}$ DCs might present alteration in antigen uptake and presentation (109), we evaluated by flow cytometry their ability to present αGalCer. Bone Marrow derived DCs (BM DCs) from wt and *was*$^{-/-}$ mice were incubated for 18 hours with αGalCer and then stained with L363 Abs (Fig.22). This analysis did not reveal any remarkable change in the presence of αGalCer-CD1d complexes on the surface of wt and *was*$^{-/-}$ BM DCs thus excluding defect in αGalCer presentation by *was*$^{-/-}$ DCs.

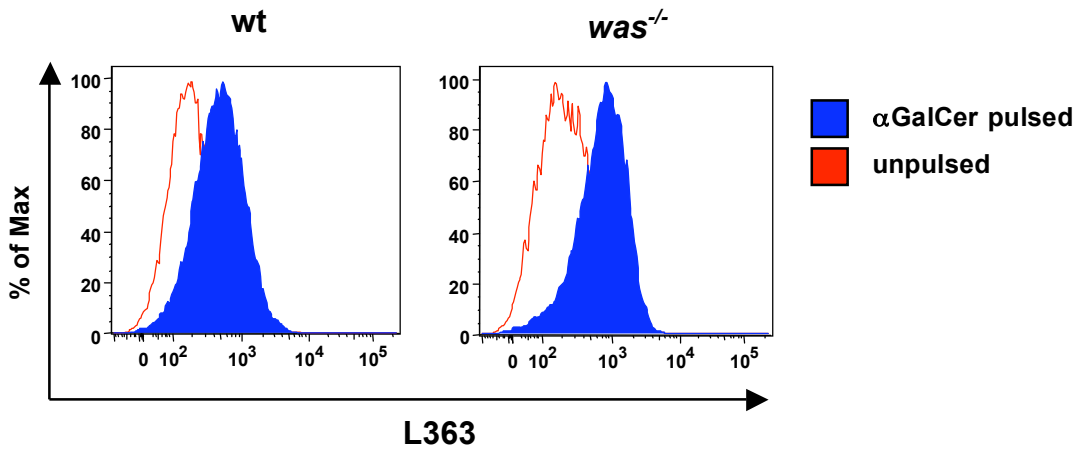

Figure 22. αGalCer presentation by *was*$^{-/-}$ DCs. BM DCs from wt and *was*$^{-/-}$ mice were incubated for 18 hours with medium alone (unpulsed) or αGalCer 100ng/ml (αGalCer pulsed) and then stained with L363 Ab. The figure shows one representative result from three independent experiments.

5.3.6 *In vitro* cytokine production ability of *was*$^{-/-}$ iNKT cells

The *in vitro* functional experiments of co-culture between DCs and iNKT cells described above suggested a cell autonomous defect in cytokine production of WASp deficient iNKT cells, especially in IL-4 production.

To further confirm the intrinsic defect of *was*$^{-/-}$ iNKT cells in cytokine production, we *in vitro* stimulated sorted wt and *was*$^{-/-}$ iNKT cells with anti-CD3 and anti-CD28 mAbs. Moreover, in order to understand whether the *was*$^{-/-}$ iNKT cell functional defect is dependent on TCR triggering, we stimulated wt and *was*$^{-/-}$ iNKT cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and the calcium ionophore Ionomycin, which activates respectively signal transduction enzyme protein kinase C bypassing TCR engagement and opens Ca^{++} channels. Following 48h of stimulation, the amount of both IL-4 and IFN-γ secreted by *was*$^{-/-}$ iNKT cells, was dramatically decreased in comparison to the wt control (Fig 23). However, upon TPA and Ionomycin stimulation, *was*$^{-/-}$ iNKT cells produced sustained cytokine levels. In addition, to further define the relevance of the TCR-induced proximal signals, we stimulated total wt and *was*$^{-/-}$ splenocytes with TPA and Ionomycin. After 1hour of incubation, Brefeldin A was added to the cells to avoid cytokine secretion and the cells were incubated for another hour. IL-4 and IFN-γ production were then measured at the single-cell level by intra-cellular FACS analysis (Fig.24A and 24B). When TCR triggering was bypassed we could detect, as expected for *was*$^{-/-}$ conventional T cells, normal percentages and MFI of IFN-γ producing *was*$^{-/-}$ iNKT cells. Upon activation with TPA/Ionomycin, *was*$^{-/-}$ iNKT cells still retained the capacity to produce detectable levels of IL-4, even if they displayed a concrete impairment in the ability to secrete IL-4 when compared to normal controls. Together these data represent a proof of the importance of TCR engagement in determining the defect of *was*$^{-/-}$ iNKT cells in producing both IFN-γ and IL-4.

An indirect measure of TCR functionality in iNKT cells is the avidity by which the semi-invariant TCR of NKT binds to the αGalCer-CD1d complex. This can be easily determined by staining iNKT cells with different concentration of CD1d tetramers loaded with PBS57, an analog of αGalCer. In line with the reduced functionality of *was*$^{-/-}$ iNKT cells, the lack of WASp leads to an impaired TCR avidity in iNKT cells, which presented a reduced capacity to bind low and high amount of the PB57-CD1d complexes in comparison with wt controls (Fig. 25).

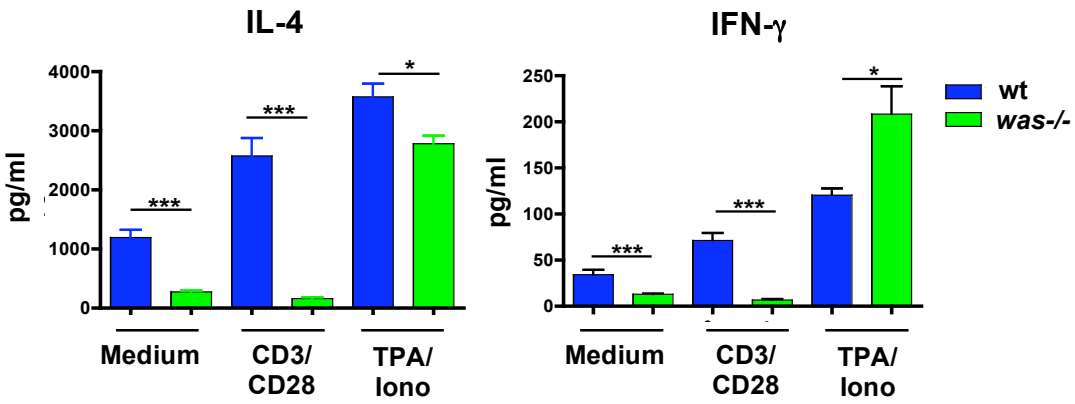

Figure 23. *In vitro* stimulation of *was*$^{-/-}$ iNKT cells with anti-CD3 and anti-CD28 mAbs. Splenic iNKT cells from wt and *was*$^{-/-}$ mice were sorted out by FACS and put in culture with medium alone or in the the presence of anti-CD3 (2μg/ml) and anti-CD28 (2μg/ml) mAbs or with medium supplemented with TPA (50 ng/ml) and Ionomycin (1μg/ml). After 48hours supernatants were collected for cytokine measurement by Bioplex technology. The graph shows the results from two independent experiments and for each experiment cells from at least 5 mice/group were pooled. Bars are mean and SEM for each group. *=$p<0.05$; *** = $p<0.001$

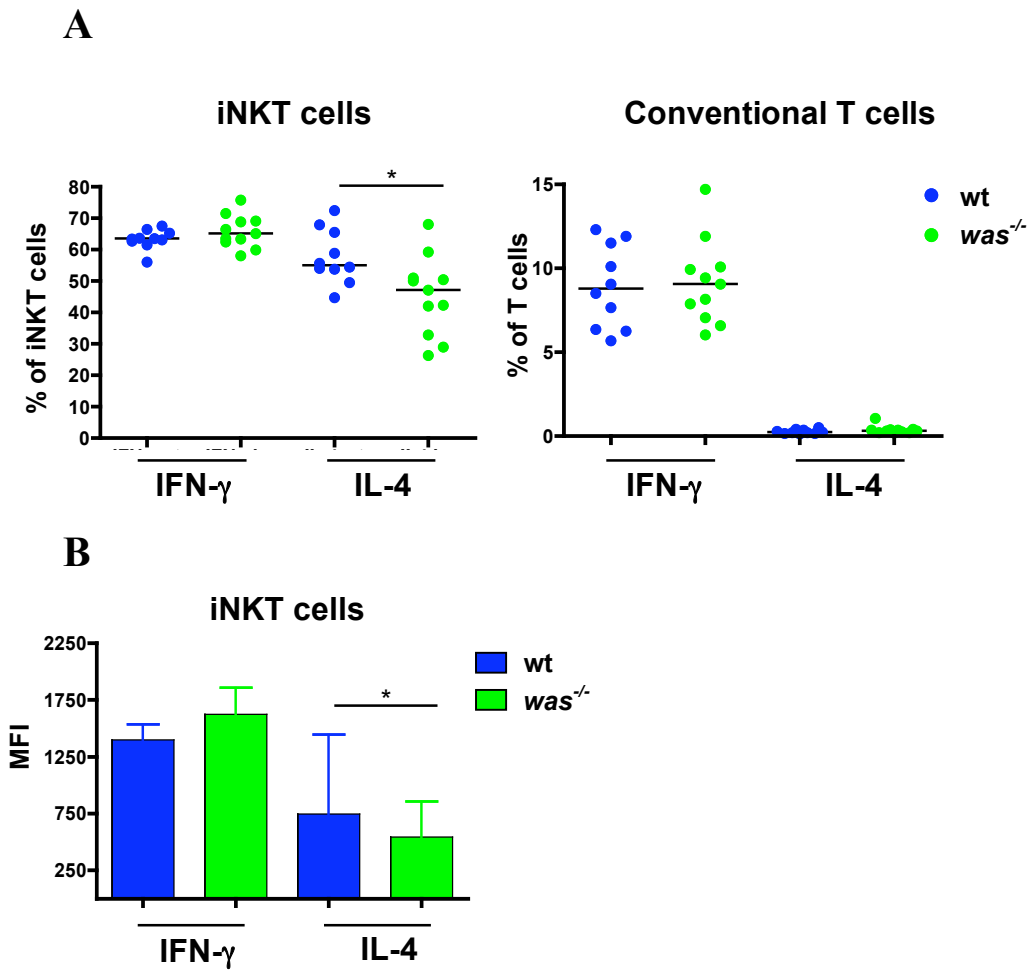

Figure 24. *In vitro* stimulation of *was*$^{-/-}$ iNKT cells with TPA and Ionomycin. Total splenocytes from wt and *was*$^{-/-}$ mice were stimulated with TPA (50 ng/ml) and Ionomycin (1μg/ml) for 2 hours at 37°C. In the last hour of incubation Brefeldin A (10 μg/ml) was added to the culture. After the stimulation cells were surface stained with anti-B220, anti-CD3 and PBS57-CD1d tetramers to detect iNKT cells. Splenocytes were then intracellularly stained with anti-IFN-γ and anti-IL-4. (A) The figure shows the percentage of IFN-γ and IL-4 producing iNKT cells (left) and conventional T cells (right) from three independent experiments. Bars are the median value for each group. (B) Among the same samples it was also measured the MFI of IFN-γ and IL-4 producing iNKT cells. Bars represent median and interquartile range. . *=$p<0.05$

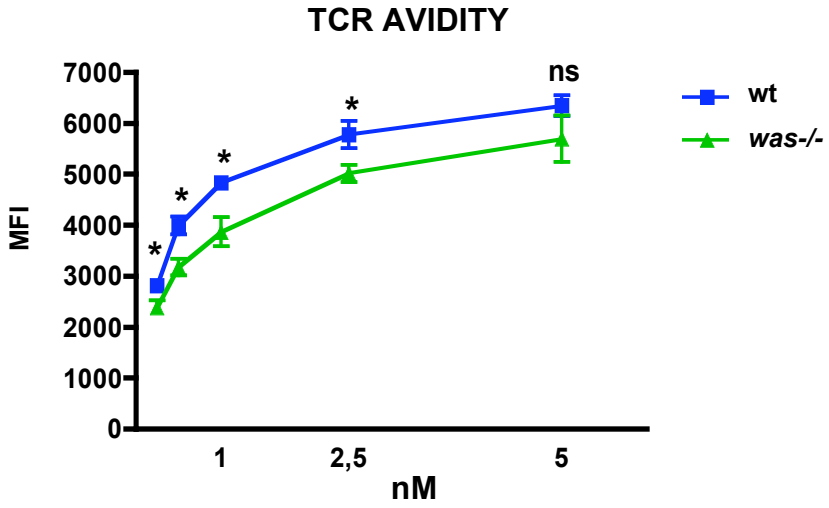

Figure 25. TCR Avidity of *was*$^{-/-}$ iNKT cells. Total splenocytes from wt and *was*$^{-/-}$ mice were incubated with different concentrations of PBS57-loaded CD1d tetramers (0.25, 0.5, 1, 2.5 and 5 nM). Cells were also stained as described in Figure legend 22 to detect iNKT cells by flow cytometry. The graph shows data from one experiment representative of three. Blue square and green triangle represent respectively the median value of wt and *was*$^{-/-}$ mice group. The vertical bars represent interquartile range of each group. *=$p<0.05$

6. DISCUSSION

The results obtained during the course of this PhD project allowed the achievement of new insight on the role of WASp in immune system regulation. We pointed out the crucial role of WASp in the biology of invariant Natural Killer T (iNKT) cells. Indeed we demonstrated for the first time that WAS patients are devoid of iNKT cells. In addition WASp is required for the normal development and tissue distribution of murine iNKT cells, as revealed by studies on *was*$^{-/-}$ mice. Similar to conventional T cells, WASp is strictly required for the effector function of iNKT cells, as *was*$^{-/-}$ iNKT cells fail to properly proliferate *in vivo* and to produce cytokine following *in vivo* and *in vitro* stimulation. Given the key role of iNKT cells in immune responses against some pathogens and in antitumoral immune responses, our data suggest that iNKT cell dysfunction might contribute to the recurrent infections and to the high tumor incidence observed in WAS patients.

6.1 WAS PATIENTS ARE DEVOID OF iNKT CELLS

Mature iNKT cells are strong immunoregulatory elements, since they promptly produce a wide range of cytokines upon TCR triggering and thus activate different immune cell type (122, 150). Thanks to this functional feature, iNKT cells are involved in the control of pathogen infection (240), in cancer immunosurveillance (150) and play a protective role in many autoimmune diseases (150, 246). In keeping with their immunoregulatory role, a complete lack of iNKT cells was found in X linked lymphoproliferative disease (XLP) (290) and in the Omenn syndrome (292), two different primary immunodeficiencies characterized, respectively, by inappropriate response to EBV infection (usually leading to B cell lymphoma), and severe autoimmune manifestations. All these evidences have prompted us to investigate iNKT cells in the Wiskott Aldrich Syndrome (WAS), a primary immunodeficiency associated with recurrent infections, increased risk to develop cancer, mainly B cell lymphoma EBV associated, and autoimmunity (3). We found that WASp deficiency dramatically impacts on the amount of circulating human iNKT cells since, by flow cytometry, iNKT cells were nearly undetectable in WAS patients with severe score (Fig. 1A). Interestingly, three patients with XLT, a milder form of WAS, presented a detectable number of iNKT cells (Fig. 1B). Therefore,

impairment in iNKT cell number correlates with the severity of disease. In severe WAS patients lacking iNKT cells, the frequency of T cells expressing either TCR Vα24 or Vβ11, although variable, was in the range of healthy donors, suggesting that these TCR V regions can be utilized by WASp deficient T cells (Fig.3). Moreover, the analysis of TCR V beta repertoire in WAS and XLT patients revealed the presence of all TCR Vβ families, with some alterations probably due to concurrent infections, frequent in these patients (Table II). Our findings are in line with previously published data showing that TCR Vβ repertoire is normal in young WAS patients and become skewed after 15 years of age (99). Thus, these data indicate a profound defect in iNKT cell population in spite of minor alterations in the TCR repertoire of mainstream T cells.

Overall, these data suggest that the absence of iNKT cells might contribute to the increased susceptibility of patients to pathogen infections. Indeed, lack of iNKT cells can cause impaired immune protection against viral, bacterial, and fungal infections. For instance, iNKT cells were demonstrated to exert a protective role in immune response against pathogens as *Streptococcus pneumoniae* and herpes simplex virus (HSV) (240). Interestingly pneumonia and HSV infection are among the most common recurring events in WAS patients (4). Moreover, as suggested for XLP, we hypothesize that the absence of iNKT cells might contribute to the high susceptibility to develop EBV^+ B cell lymphoma observed in WAS patients. iNKT cells play a key role also in tumor immunosurveillance, as suggested by the increased susceptibility to develop cancer in NKT cell deficient mice and a decreased iNKT cell number and function in patients with advanced cancer (270, 271). Thus we speculate that the increase of tumor development in WAS patients might be a consequence of the reduced functionality of WASp null NK and CD8 T cells, together with the lack of immunosurveillance operated by iNKT cells. According to this hypothesis, XLT patients, who have a detectable population of iNKT cells, are usually more resistant to infections and tumor development (4).

iNKT cells are under the level of detection in WAS patients, but this observation does not exclude the presence of few not measurable iNKT cells that still retain functional activity. To address this issue, iNKT cells from PBMCs of HDs, XLT and WAS patients, were expanded *in vitro* for several weeks (Figure 4). Following this long-term stimulation, iNKT cells from XLT and WAS patients became measurable.

However, while healthy donor iNKT cells selectively responded to the specific stimulus αGalCer becoming almost the totality of the expanded population, iNKT cells lacking normal WASp did not, as they were only a minority of the finally harvested population. In addition, the expansion did not generate a sufficient number of iNKT cells from XLT/WAS patients to perform further functional studies. Nonetheless, the lack of a specific expansion to αGalCer is by itself a proof of functional abnormality of WASp deficient iNKT cells.

Altogether these findings demonstrate the crucial role of WASp in the development/homeostasis and expansion of human iNKT cells and suggest the contribution of this cell subset in the control of infection and cancer progression.

6.2 IMPAIRED DEVELOPMENT OF MURINE was$^{-/-}$ iNKT CELLS

The lack of a detectable iNKT population in WAS patients did not allow us to establish how WASp controls the development and/or the peripheral homeostasis of human iNKT cells. Thus we moved to the *was*$^{-/-}$ murine model to better define the role of WASp in the biology of iNKT cells. Differently from humans, in mice WASp deficiency is not associated with complete lack of iNKT cells. However *was*$^{-/-}$ iNKT cell numbers were significantly reduced in thymus and in periphery (Figure 7). The requirement for WASp is similar for the different subset of iNKT cells and does not cause relevant skewing toward a particular iNKT cell type. Indeed we observed an equal reduction of CD4^{+} and DN iNKT cells in all the organs analyzed and a normal frequency of *was*$^{-/-}$ iNKT cells bearing the most representative Vβ TCRs (Figure 9 and 10).

Of note, the effect of WASp deficiency on iNKT cell frequency is more severe in periphery than in the thymus, where iNKT cells are mildly decreased in comparison to wt mice (Figure 7). This observation was confirmed by another group (296) and likely reflects inefficient egress and resultant accumulation of thymic iNKT cells. Emigration of thymic iNKT cells is regulated by S1P signaling (297), which on the other hand was shown to be mediated by WASp in marginal zone B cells (298, 299). Thus *was*$^{-/-}$ iNKT cells might present a defective S1P signaling and consequently an impaired migration in periphery. However CD69, which acts as negative regulator of this event and is downregulated upon S1P-receptor engagement (300), is normally expressed on thymic and peripheral *was*$^{-/-}$ iNKT cells (Figure 10). Astrakhan and

colleagues, claimed that *was*$^{-/-}$ iNKT cells might present a reduced migration to the liver because their lower expression of CD11a (296). However it is not clear why the reduced CD11a expression on *was*$^{-/-}$ iNKT cells should be relevant for their migration to the liver since it was demonstrated that LFA-1 (CD11a/CD18) expression on other liver cells is required for the accumulation of CD4^{+} iNKT cells (301).

A possible explanation for the reduction of iNKT cells in *was*$^{-/-}$ mice might be an altered thymic development. A normal expression of CD1d on DP lymphocytes, which is required for iNKT cell positive selection (152), was shown in *was*$^{-/-}$ mice in comparison to wild type mice (Fig. 11). In support of the hypothesis that a normal iNKT cell positive selection occurs in the thymus of *was*$^{-/-}$ mice, we did not observe gross alteration in TCR Vβ distribution of *was*$^{-/-}$ iNKT cells (Fig 10). In line with this observation, using CD44 and NK1.1 markers, we found that *was*$^{-/-}$ iNKT cells can pass through the positive selection, but are blocked in the late developmental stage 2 (CD44^{+}NK1.1^{-}) (Fig.12), suggesting a potential role of WASp in regulating the late phases of the differentiation process. This hypothesis is further supported by the expression of DX5 by thymic *was*$^{-/-}$ iNKT cells (Fig.12), since DX5 is a late maturation marker expressed before the progression from stage 2 (CD44^{+}NK1.1^{-}) to stage 3 (CD44^{+}NK1.1^{+}) (152).

Among the events contributing to the final maturation of iNKT cells in the periphery, a crucial is played by CD1d recognition, since NK1.1^{-} iNKT cells fail to properly complete their maturation in the absence of CD1d (209). Our data rule out the possibility that an altered CD1d expression in the periphery might cause the developmental block of *was*$^{-/-}$ iNKT cells (Fig.11). However, this finding does not exclude that *was*$^{-/-}$ iNKT cells might not properly interact with CD1d-expressing APCs. The lack of WASp indeed induces a cell autonomous developmental arrest, as demonstrated by the generation of mixed BM chimeras, in which *was*$^{-/-}$ iNKT cells were not able to acquire the mature phenotype, even in the presence of wt APCs (Fig.13).

Many players regulating iNKT cell terminal maturation are molecules involved in the TCR signaling. For instance a lower iNKT generation and maturation was observed in the absence of PKCθ, a signal transduction molecule known to play an important role in the TCR / NF-κB pathway (214). Of note, PKCθ–mediated phosphorylation can activate WASp at the IS (58). Thus, in iNKT cell development, WASp might be a

relevant target of the activated PKCθ in the TCR / NF-κB pathway. In addition, iNKT cell development requires TCR-induced transcriptional factors, such as NF-κB, AP-1 and T-bet (152), whose expression or function are altered in the absence of WASp (27, 76, 77). Indeed, a reduced iNKT cell generation and maturation is associated with the absence of the transcriptional factor NF-κB (215). Moreover WASp has been demonstrated, at least in NK cells, to be involved in the regulation of NF-κB nuclear translocation (77). In addition, transgenic mice over-expressing BATF, a negative regulator of AP-1 activity, show defective maturation of iNKT cells (220). Interestingly WASp absence is associated with a lower AP-1 binding activity in murine T cells (76). A severe block in NK1.1 expression is also reported in mice lacking T-bet (221), a transcriptional factor associated to Th1 immunity. A possible role for WASp in the regulation of T-bet is supported by a recent study from our group showing a reduced T-bet expression in TCR stimulated $CD4^+$ T cell lines from WAS patients (27).

Furthermore, very recent evidences have highlighted the critical role of costimulatory signals arising from B7-CD28 interaction in promoting the expansion of mature $NK1.1^+$ iNKT cells (224, 225). Interestingly, WASp was demonstrated to be required for a normal ligation-induced CD28 endocytosis, a process relevant to CD28 costimulatory functions (66). These evidences support the hypothesis that WASp acts as an important component in the downstream events of TCR pathway during iNKT cell terminal maturation.

In our *was*$^{-/-}$ murine model iNKT cells are arrested in a developmental stage characterized by a massive cellular expansion and egression from the thymus (152). Thus this late developmental block does not explain why iNKT cells, even if immature, are less abundant in the thymus and in the periphery of *was*$^{-/-}$ mice. The analysis of the *in vivo* thymic turnover revealed that *was*$^{-/-}$ iNKT cells display a proliferative rate significantly higher than wt counterpart (Fig.15). These data are consistent with the increased proportion of immature iNKT cells in *was*$^{-/-}$ mice, as $NK1.1^-$ iNKT cells are the iNKT cell fraction most actively proliferating (164). Indeed when the analysis was restricted to the immature iNKT cell fraction, the frequency of proliferating thymic iNKT cells increased from 25% to 75% in wt iNKT cells, whereas it remained around 20-40% in *was*$^{-/-}$ iNKT cells. This observation discloses a severely compromised homeostatic proliferation ability of *was*$^{-/-}$ iNKT

cells in the thymus and in the periphery as well, thus explaining the reason of the smaller *was*$^{-/-}$ iNKT cell pool.

WASp is important in driving the homeostatic process in different immune cell types. In a distinct WASp deficient murine model, Maillard and coworkers found decreased number of naturally occurring T regulatory cells (nTreg) (43), suggesting a defective nTreg homeostasis. In parallel, another group shown that signal mediated by WASp is important for nTreg peripheral expansion and survival (42). By using BrdU labeling assay, it was also highlighted the involvement of WASp in the regulation of B cell homeostasis. Mature *was*$^{-/-}$ B cells are characterized by an abnormal turnover rate but, differently from iNKT cells, they displayed an increased BrdU incorporation (298, 299), thus underlying different requirement for peripheral homeostasis in B and iNKT cells.

Among the requirement for the homeostasis of iNKT cells, IL-15 signaling plays a key role. Indeed Matsuda et al demonstrated that the proliferation and maintenance of iNKT cells is highly dependent on IL-15 (232), because IL15$^{-/-}$ mice have reduced *in vivo* turnover. Moreover IL-15 is also important for the progression from developmental stage 2 to stage 3, as revealed by the reduced maturation of iNKT cells in IL15$^{-/-}$ mice and the upregulation of IL-2/IL-15 receptor β chain (CD122) on iNKT cells when they progress through the latest developmental step (232). However, our study revealed that *was*$^{-/-}$ iNKT cells normally express CD122 and *in vitro* proliferate in response to IL-15 (Fig.16), thus ruling out a defective IL-15 signaling as the cause of the developmental block and reduced homeostatic proliferation. Supporting our results, similar findings were achieved independently in the other *was*$^{-/-}$ murine model (296).

It remains an open issue which signaling pathway, leading to the homeostatic proliferation of iNKT cells, may be normally controlled by WASp. Even if a thorough comprehension of the signaling pathways involved in the homeostatic regulation of iNKT cells still needs to be achieved, it is known that IL-7, to a lesser extent than IL-15, regulates the size of peripheral iNKT cell pool (232). However, since no data are reported in literature, is difficult to predict whether WASp is required for IL-7 induced signaling or IL-7 production.

Although TCR-CD1d interaction is crucial for the development and activation of iNKT cells, it seems to be less important for the homeostatic regulation. Indeed, there are evidences that mature iNKT cells can survive for many weeks in the absence of

CD1d (209) and that iNKT cells undergo cell divisions upon adoptive transfer in *CD1d*$^{-/-}$ mice (232).

Costimulatory signal delivered through ICOS/ICOSL interaction can contribute to the peripheral survival of these cells, and when this interaction is disrupted iNKT cells display an higher apoptosis rate (226). Another important requirement for iNKT cell homeostasis in the liver, but not in the spleen, relies on the interaction between CXCR6 on iNKT cells and CXCL16 on liver sinusoidal cells. When this interaction is interrupted, hepatic iNKT cells undergo to apoptosis to higher extent within the liver (163). Although the role of WASp in ICOS and CXCR6 signaling, has not been established yet, it seems unlikely that this signaling accounts for the abnormal turnover of *was*$^{-/-}$ iNKT cells, since we did not detect any increase in apoptotic cell death in these cells (Fig.14).

Nevertheless, many studies on iNKT cell homeostasis focused on the pool of mature iNKT cells, and thus it is not clear whether immature NK1.1^{-} iNKT cells (as the *was*$^{-/-}$ iNKT cells) can present homeostatic requirements different from those of the mature counterpart or if the reduced homeostatic proliferation of *was*$^{-/-}$ iNKT cells is related to their inability to complete the maturation process.

6.3 REDUCED FUNCTION OF MURINE *was*$^{-/-}$ iNKT CELLS

We showed that murine WASp deficient iNKT cells are impaired in the effector function upon *in vivo* activation. Indeed upon *in vivo* injection of the specific stimulus αGalCer, we detected a delayed and reduced expansion of *was*$^{-/-}$ iNKT cells (Fig. 19). Moreover we observed a lower Th1 and Th2 cytokine production in the sera of *was*$^{-/-}$ mice in comparison to wt controls following αGalCer administration (Fig.17). We demonstrated, by an *ex-vivo* analysis at the single iNKT cell level, that this reduction was not caused only by the lower number of iNKT cell in *was*$^{-/-}$ mice, but also by the impaired ability of *was*$^{-/-}$ iNKT cells to produce cytokines upon *in vivo* activation (Fig. 18). In addition, the functional deficiency of *was*$^{-/-}$ iNKT cells was further confirmed by their inability to help antigen specific B cell responses *in vivo* (Fig.20), in comparison to what wild type activated iNKT cells were shown to do (253).

The functional impairment of *was*$^{-/-}$ iNKT cells upon *in vivo* activation may not be due to their arrest at the immature stage 2 of differentiation, since peripheral wt iNKT cells with immature phenotype are able to produce both Th1 and Th2 cytokines (211).

The decreased functionality of *was*$^{-/-}$ iNKT cells upon *in vivo* activation may instead result from various factors such as alterations in antigen presentation, an improper interaction between NKT cells and APCs or a cell autonomous defect.

Indeed the activation of iNKT cells requires TCR recognition of glycolipid antigens/CD1d complex on the surface of APCs (122). It is possible that a reduced antigen presentation ability of *was*$^{-/-}$ APCs may contribute to the *in vivo* impaired functionality of *was*$^{-/-}$ iNKT cells, since *was*$^{-/-}$ DCs have a reduced ability to migrate, assemble podosomes and process particulate antigens (3). Our analysis revealed that following *in vitro* incubation, *was*$^{-/-}$ bone marrow–derived DCs are not defective in presenting on the surface the αGalCer/CD1d complex (Fig.22). However, a proper presentation of αGalCer requires the localization of the αGalCer/CD1d complex on the lipid rafts (191). Of note, the treatment of αGalCer pulsed DCs with MβCD, which disrupts lipid raft microdomain structures, markedly reduces their ability to stimulate IFN-γ production by splenocytes, whereas it has no effect on stimulation of IL-4 (191). Because of its involvement in the regulation of lipid raft dynamic (28), it seems possible that αGalCer/CD1d complex does not co-localize with lipid raft in *was*$^{-/-}$ DCs. However, further experiments are required to address this issue. Moreover, it is tempting to speculate that, like conventional T lymphocytes, iNKT cells require IS formation with an APC to achieve a proper activation. In the absence of WASp, this process has been demonstrated to be defective (28, 91) and WASp was shown to be crucial for the generation of a long-lived IS (91). Although there in no direct proof of a defective IS formation between *was*$^{-/-}$ DCs and *was*$^{-/-}$ iNKT cells, an indirect evidence of abnormal cross talk, causing the reduced IFN-γ production (Fig. 21), derives from *in vitro* studies. On the contrary, the same experiments indicate an intrinsic functional defect of *was*$^{-/-}$ iNKT cells in the pathway leading to IL-4 production. Interestingly, these data fit with the evidence discussed above, that a proper antigen presentation by DCs is required for αGalCer-induced IFN-γ, but for IL-4 production.

When sorted *was*$^{-/-}$ iNKT cells are stimulated *in vitro* with anti-CD3 and anti-CD28 mAbs, they are severely compromised in their capacity to produce both IL-4 and IFN-γ (Fig.23). However, when WASp deficient iNKT cells are activated bypassing proximal events downstream TCR triggering, they produce sustained level of both cytokines (Fig 23 and Fig.24), suggesting a TCR-mediated functional impairment of

these cells. Supporting this hypothesis *was*$^{-/-}$ iNKT cells present a decreased TCR avidity (Fig.25). Altogether these results demonstrate the relevance of WASp in TCR-mediated effector function of iNKT cells. On the other hand, important TCR-induced signaling pathways, in which WASp is involved, have been demonstrated to be crucial for the function of iNKT cells. As discussed for the development, TCR engagement activates PKCθ, which relays signals to downstream effectors and mediates WASp activation at IS (58). PKCθ was shown to be required not only for the development but also for the function of iNKT cells (214). In conventional T cells, the Tec kinase Itk plays a significant role in signaling downstream the TCR by mediating the activation NFAT and AP-1 transcriptional factors. The lack of Itk in iNKT cells leads to a severe reduction in both Th1 and Th2 cytokine production upon *in vivo* activation (223). Itk was also shown to regulate CDC42 and WASp localized activation at IS upon TCR triggering (87, 88), thus the TCR/Itk/WASp axis might be crucial in the regulation of iNKT cell function. Furthermore, altered activation of transcriptional factors, NF-κB (77) and NFAT (27) (76) occurring in the absence of WASp, may affect not only the development as discussed above, but also iNKT cell function. Indeed in iNKT cells, NF-κB regulates Th1 and Th2 cytokine production (214), whereas NFAT2 seems to control Th2 but not Th1 responses (254). Finally the Th1 master regulator T-bet, which in iNKT cells is involved in IFN-γ production (221), is defectively activated in the absence of WASp, as demonstrated by the reduced T-bet expression in TCR-stimulated CD4 T cells from WAS patients (27).

6.4 CONCLUDING REMARKS

Overall, these results show that the lack of WASp leads to profound alterations in iNKT cells, which are absent in patients with severe clinical phenotype and severely reduced in the *was*$^{-/-}$ mouse model. In *was*$^{-/-}$ mice, accumulation of immature iNKT cells ($CD44^{+}$ $NK1.1^{-}$), together with the lack of mature subset, suggests a key role for WASp in iNKT cell maturation process. Moreover, analysis of *in vivo* and *in vitro* stimulation and cytokine production reveals that peripheral iNKT cells are functionally impaired in *was*$^{-/-}$ mice. WASp is known as a central player in T cell activation by controlling actin polymerization, which in turn favors the generation of a long lived IS between T cells and APCs (28, 91). We hypothesize that the differentiation process and the effector function of iNKT cells require the formation

of a proper IS between iNKT cells and CD1d-expressing cells. The stable IS may allow iNKT cells to receive and integrate various agonist signals, originating from invariant TCR engagement and co-stimulatory molecules in order to achieve full differentiation and proper functional activity. Indeed, the defective *was*$^{-/-}$ iNKT cells resemble the ones generated in the absence of signal transduction molecules or transcription factors, such as PKCθ, NF-κB, or T-bet (214, 215, 221). These molecules belong to the complex cascade of events generated upon TCR triggering and IS formation in conventional T cells, and regulate iNKT cell expansion or survival, maturation and cytokine production. Furthermore, the genetic deletion of costimulatory molecules such as CD28 affects expansion and phenotypic and functional differentiation of iNKT cells (224, 225).

In conclusion, these findings provide the first evidence that WASp acts as an important player for the generation of mature and functional iNKT cells. Moreover, our data add a new perspective in the comprehension of the complex immune dysregulation and tumor susceptibility characterizing the Wiskott Aldrich syndrome.

7. BIBLIOGRAPHY

1. Wiskott, A. 1937. Familiarer, angeborener morbus Werlhofii? *Mschr. Kinderheilk* 68:212-216.
2. Aldrich, R.A., A.G. Steinberg, and D.C. Campbell. 1954. Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhea. *Pediatrics* 13:133-139.
3. Ochs, H.D., and A.J. Thrasher. 2006. The Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* 117:725-738; quiz 739.
4. Imai, K., T. Morio, Y. Zhu, Y. Jin, S. Itoh, M. Kajiwara, J. Yata, S. Mizutani, H.D. Ochs, and S. Nonoyama. 2004. Clinical course of patients with WASP gene mutations. *Blood* 103:456-464.
5. Derry, J.M., H.D. Ochs, and U. Francke. 1994. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* 78:635-644.
6. Stewart, D.M., S. Treiber-Held, C.C. Kurman, F. Facchetti, L.D. Notarangelo, and D.L. Nelson. 1996. Studies of the expression of the Wiskott-Aldrich syndrome protein. *J Clin Invest* 97:2627-2634.
7. Symons, M., J.M. Derry, B. Karlak, S. Jiang, V. Lemahieu, F. McCormick, U. Francke, and A. Abo. 1996. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell* 84:723-734.
8. Villa, A., L. Notarangelo, P. Macchi, E. Mantuano, G. Cavagni, D. Brugnoni, D. Strina, M.C. Patrosso, U. Ramenghi, M.G. Sacco, and et al. 1995. X-linked thrombocytopenia and Wiskott-Aldrich syndrome are allelic diseases with mutations in the WASP gene. *Nat Genet* 9:414-417.
9. Notarangelo, L.D., C. Mazza, S. Giliani, C. D'Aria, F. Gandellini, C. Ravelli, M.G. Locatelli, D.L. Nelson, H.D. Ochs, and L.D. Notarangelo. 2002. Missense mutations of the WASP gene cause intermittent X-linked thrombocytopenia. *Blood* 99:2268-2269.
10. Devriendt, K., A.S. Kim, G. Mathijs, S.G. Frints, M. Schwartz, J.J. Van Den Oord, G.E. Verhoef, M.A. Boogaerts, J.P. Fryns, D. You, M.K. Rosen, and P. Vandenberghe. 2001. Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. *Nat Genet* 27:313-317.
11. Ancliff, P.J., M.P. Blundell, G.O. Cory, Y. Calle, A. Worth, H. Kempski, S. Burns, G.E. Jones, J. Sinclair, C. Kinnon, I.M. Hann, R.E. Gale, D.C. Linch, and A.J. Thrasher. 2006. Two novel activating mutations in the Wiskott-Aldrich syndrome protein result in congenital neutropenia. *Blood* 108:2182-2189.
12. Arkwright, P.D., M. Abinun, and A.J. Cant. 2002. Autoimmunity in human primary immunodeficiency diseases. *Blood* 99:2694-2702.
13. Shcherbina, A., F.S. Rosen, and E. Remold-O'Donnell. 1999. WASP levels in platelets and lymphocytes of wiskott-aldrich syndrome patients correlate with cell dysfunction. *J Immunol* 163:6314-6320.
14. Ochs, H.D., S.J. Slichter, L.A. Harker, W.E. Von Behrens, R.A. Clark, and R.J. Wedgwood. 1980. The Wiskott-Aldrich syndrome: studies of lymphocytes, granulocytes, and platelets. *Blood* 55:243-252.
15. Kajiwara, M., S. Nonoyama, M. Eguchi, T. Morio, K. Imai, H. Okawa, M. Kaneko, M. Sako, S. Ohga, M. Maeda, S. Hibi, H. Hashimito, A. Shibuya, H.D. Ochs, T. Nakahata, and J.I. Yata. 1999. WASP is involved in

proliferation and differentiation of human haemopoietic progenitors in vitro. *Br J Haematol* 107:254-262.

16. Haddad, E., E. Cramer, C. Riviere, P. Rameau, F. Louache, J. Guichard, D.L. Nelson, A. Fischer, W. Vainchenker, and N. Debili. 1999. The thrombocytopenia of Wiskott Aldrich syndrome is not related to a defect in proplatelet formation. *Blood* 94:509-518.
17. Sabri, S., A. Foudi, S. Boukour, B. Franc, S. Charrier, M. Jandrot-Perrus, R.W. Farndale, A. Jalil, M.P. Blundell, E.M. Cramer, F. Louache, N. Debili, A.J. Thrasher, and W. Vainchenker. 2006. Deficiency in the Wiskott-Aldrich protein induces premature proplatelet formation and platelet production in the bone marrow compartment. *Blood* 108:134-140.
18. Grottum, K.A., T. Hovig, H. Holmsen, A.F. Abrahamsen, M. Jeremic, and M. Seip. 1969. Wiskott-Aldrich syndrome: qualitative platelet defects and short platelet survival. *Br J Haematol* 17:373-388.
19. Murphy, S., F.A. Oski, J.L. Naiman, C.J. Lusch, S. Goldberg, and F.H. Gardner. 1972. Platelet size and kinetics in hereditary and acquired thrombocytopenia. *N Engl J Med* 286:499-504.
20. Shcherbina, A., F.S. Rosen, and E. Remold-O'Donnell. 1999. Pathological events in platelets of Wiskott-Aldrich syndrome patients. *Br J Haematol* 106:875-883.
21. Corash, L., B. Shafer, and R.M. Blaese. 1985. Platelet-associated immunoglobulin, platelet size, and the effect of splenectomy in the Wiskott-Aldrich syndrome. *Blood* 65:1439-1443.
22. Semple, J.W., K.A. Siminovitch, M. Mody, Y. Milev, A.H. Lazarus, J.F. Wright, and J. Freedman. 1997. Flow cytometric analysis of platelets from children with the Wiskott-Aldrich syndrome reveals defects in platelet development, activation and structure. *Br J Haematol* 97:747-754.
23. Mullen, C.A., K.D. Anderson, and R.M. Blaese. 1993. Splenectomy and/or bone marrow transplantation in the management of the Wiskott-Aldrich syndrome: long-term follow-up of 62 cases. *Blood* 82:2961-2966.
24. Dupuis-Girod, S., J. Medioni, E. Haddad, P. Quartier, M. Cavazzana-Calvo, F. Le Deist, G. de Saint Basile, J. Delaunay, K. Schwarz, J.L. Casanova, S. Blanche, and A. Fischer. 2003. Autoimmunity in Wiskott-Aldrich syndrome: risk factors, clinical features, and outcome in a single-center cohort of 55 patients. *Pediatrics* 111:e622-627.
25. Tsuboi, S., S. Nonoyama, and H.D. Ochs. 2006. Wiskott-Aldrich syndrome protein is involved in alphaIIb beta3-mediated cell adhesion. *EMBO Rep* 7:506-511.
26. Sullivan, K.E., C.A. Mullen, R.M. Blaese, and J.A. Winkelstein. 1994. A multiinstitutional survey of the Wiskott-Aldrich syndrome. *J Pediatr* 125:876-885.
27. Trifari, S., G. Sitia, A. Aiuti, S. Scaramuzza, F. Marangoni, L.G. Guidotti, S. Martino, P. Saracco, L.D. Notarangelo, M.G. Roncarolo, and L. Dupre. 2006. Defective Th1 cytokine gene transcription in CD4+ and CD8+ T cells from Wiskott-Aldrich syndrome patients. *J Immunol* 177:7451-7461.
28. Dupre, L., A. Aiuti, S. Trifari, S. Martino, P. Saracco, C. Bordignon, and M.G. Roncarolo. 2002. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity* 17:157-166.

29. Molina, I.J., J. Sancho, C. Terhorst, F.S. Rosen, and E. Remold-O'Donnell. 1993. T cells of patients with the Wiskott-Aldrich syndrome have a restricted defect in proliferative responses. *J Immunol* 151:4383-4390.
30. Gismondi, A., L. Cifaldi, C. Mazza, S. Giliani, S. Parolini, S. Morrone, J. Jacobelli, E. Bandiera, L. Notarangelo, and A. Santoni. 2004. Impaired natural and CD16-mediated NK cell cytotoxicity in patients with WAS and XLT: ability of IL-2 to correct NK cell functional defect. *Blood* 104:436-443.
31. Orange, J.S., N. Ramesh, E. Remold-O'Donnell, Y. Sasahara, L. Koopman, M. Byrne, F.A. Bonilla, F.S. Rosen, R.S. Geha, and J.L. Strominger. 2002. Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proc Natl Acad Sci U S A* 99:11351-11356.
32. Westerberg, L., M. Larsson, S.J. Hardy, C. Fernandez, A.J. Thrasher, and E. Severinson. 2005. Wiskott-Aldrich syndrome protein deficiency leads to reduced B-cell adhesion, migration, and homing, and a delayed humoral immune response. *Blood* 105:1144-1152.
33. Leverrier, Y., R. Lorenzi, M.P. Blundell, P. Brickell, C. Kinnon, A.J. Ridley, and A.J. Thrasher. 2001. Cutting edge: the Wiskott-Aldrich syndrome protein is required for efficient phagocytosis of apoptotic cells. *J Immunol* 166:4831-4834.
34. Lorenzi, R., P.M. Brickell, D.R. Katz, C. Kinnon, and A.J. Thrasher. 2000. Wiskott-Aldrich syndrome protein is necessary for efficient IgG-mediated phagocytosis. *Blood* 95:2943-2946.
35. Westerberg, L., R.P. Wallin, G. Greicius, H.G. Ljunggren, and E. Severinson. 2003. Efficient antigen presentation of soluble, but not particulate, antigen in the absence of Wiskott-Aldrich syndrome protein. *Immunology* 109:384-391.
36. Zhang, H., U.Y. Schaff, C.E. Green, H. Chen, M.R. Sarantos, Y. Hu, D. Wara, S.I. Simon, and C.A. Lowell. 2006. Impaired integrin-dependent function in Wiskott-Aldrich syndrome protein-deficient murine and human neutrophils. *Immunity* 25:285-295.
37. Lacout, C., E. Haddad, S. Sabri, F. Svinarchouk, L. Garcon, C. Capron, A. Foudi, R. Mzali, S.B. Snapper, F. Louache, W. Vainchenker, and D. Dumenil. 2003. A defect in hematopoietic stem cell migration explains the nonrandom X-chromosome inactivation in carriers of Wiskott-Aldrich syndrome. *Blood* 102:1282-1289.
38. Snapper, S.B., P. Meelu, D. Nguyen, B.M. Stockton, P. Bozza, F.W. Alt, F.S. Rosen, U.H. von Andrian, and C. Klein. 2005. WASP deficiency leads to global defects of directed leukocyte migration in vitro and in vivo. *J Leukoc Biol* 77:993-998.
39. Park, J.Y., M. Kob, A.P. Prodeus, F.S. Rosen, A. Shcherbina, and E. Remold-O'Donnell. 2004. Early deficit of lymphocytes in Wiskott-Aldrich syndrome: possible role of WASP in human lymphocyte maturation. *Clin Exp Immunol* 136:104-110.
40. Trifari, S. 2007. Current understanding of the Wiskott–Aldrich syndrome and prospects for gene therapy. *Expert Review of Clinical Immunology* Vol. 3, No. 2: 205-215
41. Adriani, M., J. Aoki, R. Horai, A.M. Thornton, A. Konno, M. Kirby, S.M. Anderson, R.M. Siegel, F. Candotti, and P.L. Schwartzberg. 2007. Impaired in vitro regulatory T cell function associated with Wiskott-Aldrich syndrome. *Clin Immunol* 124:41-48.

42. Humblet-Baron, S., B. Sather, S. Anover, S. Becker-Herman, D.J. Kasprowicz, S. Khim, T. Nguyen, K. Hudkins-Loya, C.E. Alpers, S.F. Ziegler, H. Ochs, T. Torgerson, D.J. Campbell, and D.J. Rawlings. 2007. Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. *J Clin Invest* 117:407-418.
43. Maillard, M.H., V. Cotta-de-Almeida, F. Takeshima, D.D. Nguyen, P. Michetti, C. Nagler, A.K. Bhan, and S.B. Snapper. 2007. The Wiskott-Aldrich syndrome protein is required for the function of CD4(+)CD25(+)Foxp3(+) regulatory T cells. *J Exp Med* 204:381-391.
44. Marangoni, F., S. Trifari, S. Scaramuzza, C. Panaroni, S. Martino, L.D. Notarangelo, Z. Baz, A. Metin, F. Cattaneo, A. Villa, A. Aiuti, M. Battaglia, M.G. Roncarolo, and L. Dupre. 2007. WASP regulates suppressor activity of human and murine CD4(+)CD25(+)FOXP3(+) natural regulatory T cells. *J Exp Med* 204:369-380.
45. Shcherbina, A., F. Candotti, F.S. Rosen, and E. Remold-O'Donnell. 2003. High incidence of lymphomas in a subgroup of Wiskott-Aldrich syndrome patients. *Br J Haematol* 121:529-530.
46. Moulding, D.A., M.P. Blundell, D.G. Spiller, M.R. White, G.O. Cory, Y. Calle, H. Kempski, J. Sinclair, P.J. Ancliff, C. Kinnon, G.E. Jones, and A.J. Thrasher. 2007. Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med* 204:2213-2224.
47. Zhu, Q., C. Watanabe, T. Liu, D. Hollenbaugh, R.M. Blaese, S.B. Kanner, A. Aruffo, and H.D. Ochs. 1997. Wiskott-Aldrich syndrome/X-linked thrombocytopenia: WASP gene mutations, protein expression, and phenotype. *Blood* 90:2680-2689.
48. Kwan, S.P., T. Lehner, T. Hagemann, B. Lu, M. Blaese, H. Ochs, R. Wedgwood, J. Ott, I.W. Craig, and F.S. Rosen. 1991. Localization of the gene for the Wiskott-Aldrich syndrome between two flanking markers, TIMP and DXS255, on Xp11.22-Xp11.3. *Genomics* 10:29-33.
49. Zhang, J., A. Shehabeldin, L.A. da Cruz, J. Butler, A.K. Somani, M. McGavin, I. Kozieradzki, A.O. dos Santos, A. Nagy, S. Grinstein, J.M. Penninger, and K.A. Siminovitch. 1999. Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J Exp Med* 190:1329-1342.
50. Snapper, S.B., F.S. Rosen, E. Mizoguchi, P. Cohen, W. Khan, C.H. Liu, T.L. Hagemann, S.P. Kwan, R. Ferrini, L. Davidson, A.K. Bhan, and F.W. Alt. 1998. Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* 9:81-91.
51. Andreansky, S., H. Liu, S. Turner, J.A. McCullers, R. Lang, R. Rutschman, P.C. Doherty, P.J. Murray, A.W. Nienhuis, and T.S. Strom. 2005. WASP-mice exhibit defective immune responses to influenza A virus, Streptococcus pneumoniae, and Mycobacterium bovis BCG. *Exp Hematol* 33:443-451.
52. Takenawa, T., and S. Suetsugu. 2007. The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol* 8:37-48.
53. Cotta-de-Almeida, V., L. Westerberg, M.H. Maillard, D. Onaldi, H. Wachtel, P. Meelu, U.I. Chung, R. Xavier, F.W. Alt, and S.B. Snapper. 2007. Wiskott Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. *Proc Natl Acad Sci U S A* 104:15424-15429.
54. Chou, H.C., I.M. Anton, M.R. Holt, C. Curcio, S. Lanzardo, A. Worth, S. Burns, A.J. Thrasher, G.E. Jones, and Y. Calle. 2006. WIP regulates the

stability and localization of WASP to podosomes in migrating dendritic cells. *Curr Biol* 16:2337-2344.
55. de la Fuente, M.A., Y. Sasahara, M. Calamito, I.M. Anton, A. Elkhal, M.D. Gallego, K. Suresh, K. Siminovitch, H.D. Ochs, K.C. Anderson, F.S. Rosen, R.S. Geha, and N. Ramesh. 2007. WIP is a chaperone for Wiskott-Aldrich syndrome protein (WASP). *Proc Natl Acad Sci U S A* 104:926-931.
56. Konno, A., M. Kirby, S.A. Anderson, P.L. Schwartzberg, and F. Candotti. 2007. The expression of Wiskott-Aldrich syndrome protein (WASP) is dependent on WASP-interacting protein (WIP). *Int Immunol* 19:185-192.
57. Martinez-Quiles, N., R. Rohatgi, I.M. Anton, M. Medina, S.P. Saville, H. Miki, H. Yamaguchi, T. Takenawa, J.H. Hartwig, R.S. Geha, and N. Ramesh. 2001. WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat Cell Biol* 3:484-491.
58. Sasahara, Y., R. Rachid, M.J. Byrne, M.A. de la Fuente, R.T. Abraham, N. Ramesh, and R.S. Geha. 2002. Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol Cell* 10:1269-1281.
59. Dong, X., G. Patino-Lopez, F. Candotti, and S. Shaw. 2007. Structure-function analysis of the WIP role in T cell receptor-stimulated NFAT activation: evidence that WIP-WASP dissociation is not required and that the WIP NH2 terminus is inhibitory. *J Biol Chem* 282:30303-30310.
60. Thrasher, A.J. 2002. WASp in immune-system organization and function. *Nat Rev Immunol* 2:635-646.
61. Kim, A.S., L.T. Kakalis, N. Abdul-Manan, G.A. Liu, and M.K. Rosen. 2000. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* 404:151-158.
62. Higgs, H.N., and T.D. Pollard. 2000. Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J Cell Biol* 150:1311-1320.
63. Rohatgi, R., P. Nollau, H.Y. Ho, M.W. Kirschner, and B.J. Mayer. 2001. Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem* 276:26448-26452.
64. Ho, H.Y., R. Rohatgi, A.M. Lebensohn, M. Le, J. Li, S.P. Gygi, and M.W. Kirschner. 2004. Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. *Cell* 118:203-216.
65. McGavin, M.K., K. Badour, L.A. Hardy, T.J. Kubiseski, J. Zhang, and K.A. Siminovitch. 2001. The intersectin 2 adaptor links Wiskott Aldrich Syndrome protein (WASp)-mediated actin polymerization to T cell antigen receptor endocytosis. *J Exp Med* 194:1777-1787.
66. Badour, K., M.K. McGavin, J. Zhang, S. Freeman, C. Vieira, D. Filipp, M. Julius, G.B. Mills, and K.A. Siminovitch. 2007. Interaction of the Wiskott-Aldrich syndrome protein with sorting nexin 9 is required for CD28 endocytosis and cosignaling in T cells. *Proc Natl Acad Sci U S A* 104:1593-1598.
67. Tian, L., D.L. Nelson, and D.M. Stewart. 2000. Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules. *J Biol Chem* 275:7854-7861.

68. Castellano, F., C. Le Clainche, D. Patin, M.F. Carlier, and P. Chavrier. 2001. A WASp-VASP complex regulates actin polymerization at the plasma membrane. *Embo J* 20:5603-5614.
69. Badour, K., J. Zhang, F. Shi, Y. Leng, M. Collins, and K.A. Siminovitch. 2004. Fyn and PTP-PEST-mediated regulation of Wiskott-Aldrich syndrome protein (WASp) tyrosine phosphorylation is required for coupling T cell antigen receptor engagement to WASp effector function and T cell activation. *J Exp Med* 199:99-112.
70. Baba, Y., S. Nonoyama, M. Matsushita, T. Yamadori, S. Hashimoto, K. Imai, S. Arai, T. Kunikata, M. Kurimoto, T. Kurosaki, H.D. Ochs, J. Yata, T. Kishimoto, and S. Tsukada. 1999. Involvement of wiskott-aldrich syndrome protein in B-cell cytoplasmic tyrosine kinase pathway. *Blood* 93:2003-2012.
71. Cory, G.O., R. Garg, R. Cramer, and A.J. Ridley. 2002. Phosphorylation of tyrosine 291 enhances the ability of WASp to stimulate actin polymerization and filopodium formation. Wiskott-Aldrich Syndrome protein. *J Biol Chem* 277:45115-45121.
72. Torres, E., and M.K. Rosen. 2003. Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. *Mol Cell* 11:1215-1227.
73. Blundell, M.P., G. Bouma, J. Metelo, A. Worth, Y. Calle, L.A. Cowell, L.S. Westerberg, D.A. Moulding, S. Mirando, C. Kinnon, G.O. Cory, G.E. Jones, S.B. Snapper, S.O. Burns, and A.J. Thrasher. 2009. Phosphorylation of WASp is a key regulator of activity and stability in vivo. *Proc Natl Acad Sci U S A* 106:15738-15743.
74. Cory, G.O., R. Cramer, L. Blanchoin, and A.J. Ridley. 2003. Phosphorylation of the WASP-VCA domain increases its affinity for the Arp2/3 complex and enhances actin polymerization by WASP. *Mol Cell* 11:1229-1239.
75. Silvin, C., B. Belisle, and A. Abo. 2001. A role for Wiskott-Aldrich syndrome protein in T-cell receptor-mediated transcriptional activation independent of actin polymerization. *J Biol Chem* 276:21450-21457.
76. Cianferoni, A., M. Massaad, S. Feske, M.A. de la Fuente, L. Gallego, N. Ramesh, and R.S. Geha. 2005. Defective nuclear translocation of nuclear factor of activated T cells and extracellular signal-regulated kinase underlies deficient IL-2 gene expression in Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* 116:1364-1371.
77. Huang, W., H.D. Ochs, B. Dupont, and Y.M. Vyas. 2005. The Wiskott-Aldrich syndrome protein regulates nuclear translocation of NFAT2 and NF-kappa B (RelA) independently of its role in filamentous actin polymerization and actin cytoskeletal rearrangement. *J Immunol* 174:2602-2611.
78. Lutskiy, M.I., F.S. Rosen, and E. Remold-O'Donnell. 2005. Genotype-proteotype linkage in the Wiskott-Aldrich syndrome. *J Immunol* 175:1329-1336.
79. Jin, Y., C. Mazza, J.R. Christie, S. Giliani, M. Fiorini, P. Mella, F. Gandellini, D.M. Stewart, Q. Zhu, D.L. Nelson, L.D. Notarangelo, and H.D. Ochs. 2004. Mutations of the Wiskott-Aldrich Syndrome Protein (WASP): hotspots, effect on transcription, and translation and phenotype/genotype correlation. *Blood* 104:4010-4019.
80. Notarangelo, L.D., L.D. Notarangelo, and H.D. Ochs. 2005. WASP and the phenotypic range associated with deficiency. *Curr Opin Allergy Clin Immunol* 5:485-490.

81. Parolini, O., S. Berardelli, E. Riedl, C. Bello-Fernandez, H. Strobl, O. Majdic, and W. Knapp. 1997. Expression of Wiskott-Aldrich syndrome protein (WASP) gene during hematopoietic differentiation. *Blood* 90:70-75.
82. Charrier, S., L. Dupre, S. Scaramuzza, L. Jeanson-Leh, M.P. Blundell, O. Danos, F. Cattaneo, A. Aiuti, R. Eckenberg, A.J. Thrasher, M.G. Roncarolo, and A. Galy. 2007. Lentiviral vectors targeting WASp expression to hematopoietic cells, efficiently transduce and correct cells from WAS patients. *Gene Ther* 14:415-428.
83. Wengler, G., J.B. Gorlin, J.M. Williamson, F.S. Rosen, and D.H. Bing. 1995. Nonrandom inactivation of the X chromosome in early lineage hematopoietic cells in carriers of Wiskott-Aldrich syndrome. *Blood* 85:2471-2477.
84. Gallego, M.D., M. Santamaria, J. Pena, and I.J. Molina. 1997. Defective actin reorganization and polymerization of Wiskott-Aldrich T cells in response to CD3-mediated stimulation. *Blood* 90:3089-3097.
85. Badour, K., J. Zhang, F. Shi, M.K. McGavin, V. Rampersad, L.A. Hardy, D. Field, and K.A. Siminovitch. 2003. The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. *Immunity* 18:141-154.
86. Zeng, R., J.L. Cannon, R.T. Abraham, M. Way, D.D. Billadeau, J. Bubeck-Wardenberg, and J.K. Burkhardt. 2003. SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *J Immunol* 171:1360-1368.
87. Labno, C.M., C.M. Lewis, D. You, D.W. Leung, A. Takesono, N. Kamberos, A. Seth, L.D. Finkelstein, M.K. Rosen, P.L. Schwartzberg, and J.K. Burkhardt. 2003. Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr Biol* 13:1619-1624.
88. Dombroski, D., R.A. Houghtling, C.M. Labno, P. Precht, A. Takesono, N.J. Caplen, D.D. Billadeau, R.L. Wange, J.K. Burkhardt, and P.L. Schwartzberg. 2005. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J Immunol* 174:1385-1392.
89. Cannon, J.L., C.M. Labno, G. Bosco, A. Seth, M.H. McGavin, K.A. Siminovitch, M.K. Rosen, and J.K. Burkhardt. 2001. Wasp recruitment to the T cell:APC contact site occurs independently of Cdc42 activation. *Immunity* 15:249-259.
90. Cannon, J.L., and J.K. Burkhardt. 2004. Differential roles for Wiskott-Aldrich syndrome protein in immune synapse formation and IL-2 production. *J Immunol* 173:1658-1662.
91. Sims, T.N., T.J. Soos, H.S. Xenias, B. Dubin-Thaler, J.M. Hofman, J.C. Waite, T.O. Cameron, V.K. Thomas, R. Varma, C.H. Wiggins, M.P. Sheetz, D.R. Littman, and M.L. Dustin. 2007. Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. *Cell* 129:773-785.
92. Morales-Tirado, V., S. Johannson, E. Hanson, A. Howell, J. Zhang, K.A. Siminovitch, and D.J. Fowell. 2004. Cutting edge: selective requirement for the Wiskott-Aldrich syndrome protein in cytokine, but not chemokine, secretion by CD4+ T cells. *J Immunol* 173:726-730.

93. Morales-Tirado, V., D.K. Sojka, S.D. Katzman, C.A. Lazarski, F.D. Finkelman, J.F. Urban, and D.J. Fowell. 2009. Critical requirement for the Wiskott-Aldrich syndrome protein in Th2 effector function. *Blood*
94. Haddad, E., J.L. Zugaza, F. Louache, N. Debili, C. Crouin, K. Schwarz, A. Fischer, W. Vainchenker, and J. Bertoglio. 2001. The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. *Blood* 97:33-38.
95. Gallego, M.D., M.A. de la Fuente, I.M. Anton, S. Snapper, R. Fuhlbrigge, and R.S. Geha. 2006. WIP and WASP play complementary roles in T cell homing and chemotaxis to SDF-1alpha. *Int Immunol* 18:221-232.
96. Cooper, M.D., H.P. Chae, J.T. Lowman, W. Krivit, and R.A. Good. 1968. Wiskott-Aldrich syndrome. An immunologic deficiency disease involving the afferent limb of immunity. *Am J Med* 44:499-513.
97. Wolff, J.A. 1967. Wiskott-Aldrich syndrome: clinical, immunologic, and pathologic observations. *J Pediatr* 70:221-232.
98. Zhang, J., F. Shi, K. Badour, Y. Deng, M.K. McGavin, and K.A. Siminovitch. 2002. WASp verprolin homology, cofilin homology, and acidic region domain-mediated actin polymerization is required for T cell development. *Proc Natl Acad Sci U S A* 99:2240-2245.
99. Wada, T., S.H. Schurman, E.K. Garabedian, A. Yachie, and F. Candotti. 2005. Analysis of T-cell repertoire diversity in Wiskott-Aldrich syndrome. *Blood* 106:3895-3897.
100. Rawlings, S.L., G.M. Crooks, D. Bockstoce, L.W. Barsky, R. Parkman, and K.I. Weinberg. 1999. Spontaneous apoptosis in lymphocytes from patients with Wiskott-Aldrich syndrome: correlation of accelerated cell death and attenuated bcl-2 expression. *Blood* 94:3872-3882.
101. Rengan, R., H.D. Ochs, L.I. Sweet, M.L. Keil, W.T. Gunning, N.A. Lachant, L.A. Boxer, and G.M. Omann. 2000. Actin cytoskeletal function is spared, but apoptosis is increased, in WAS patient hematopoietic cells. *Blood* 95:1283-1292.
102. Burns, S., G.O. Cory, W. Vainchenker, and A.J. Thrasher. 2004. Mechanisms of WASp-mediated hematologic and immunologic disease. *Blood* 104:3454-3462.
103. Simon, H.U., G.B. Mills, S. Hashimoto, and K.A. Siminovitch. 1992. Evidence for defective transmembrane signaling in B cells from patients with Wiskott-Aldrich syndrome. *J Clin Invest* 90:1396-1405.
104. Henriquez, N.V., G.T. Rijkers, and B.J. Zegers. 1994. Antigen receptor-mediated transmembrane signaling in Wiskott-Aldrich syndrome. *J Immunol* 153:395-399.
105. Park, J.Y., A. Shcherbina, F.S. Rosen, A.P. Prodeus, and E. Remold-O'Donnell. 2005. Phenotypic perturbation of B cells in the Wiskott-Aldrich syndrome. *Clin Exp Immunol* 139:297-305.
106. Westerberg, L., G. Greicius, S.B. Snapper, P. Aspenstrom, and E. Severinson. 2001. Cdc42, Rac1, and the Wiskott-Aldrich syndrome protein are involved in the cytoskeletal regulation of B lymphocytes. *Blood* 98:1086-1094.
107. Facchetti, F., L. Blanzuoli, W. Vermi, L.D. Notarangelo, S. Giliani, M. Fiorini, A. Fasth, D.M. Stewart, and D.L. Nelson. 1998. Defective actin polymerization in EBV-transformed B-cell lines from patients with the Wiskott-Aldrich syndrome. *J Pathol* 185:99-107.

108. Vermi, W., L. Blanzuoli, M.D. Kraus, P. Grigolato, F. Donato, G. Loffredo, C.E. Marino, D. Alberti, L.D. Notarangelo, and F. Facchetti. 1999. The spleen in the Wiskott-Aldrich syndrome: histopathologic abnormalities of the white pulp correlate with the clinical phenotype of the disease. *Am J Surg Pathol* 23:182-191.
109. Bosticardo, M., F. Marangoni, A. Aiuti, A. Villa, and M. Grazia Roncarolo. 2009. Recent advances in understanding the pathophysiology of Wiskott-Aldrich syndrome. *Blood* 113:6288-6295.
110. Borg, C., A. Jalil, D. Laderach, K. Maruyama, H. Wakasugi, S. Charrier, B. Ryffel, A. Cambi, C. Figdor, W. Vainchenker, A. Galy, A. Caignard, and L. Zitvogel. 2004. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. *Blood* 104:3267-3275.
111. Badolato, R., S. Sozzani, F. Malacarne, S. Bresciani, M. Fiorini, A. Borsatti, A. Albertini, A. Mantovani, A.G. Ugazio, and L.D. Notarangelo. 1998. Monocytes from Wiskott-Aldrich patients display reduced chemotaxis and lack of cell polarization in response to monocyte chemoattractant protein-1 and formyl-methionyl-leucyl-phenylalanine. *J Immunol* 161:1026-1033.
112. Zicha, D., W.E. Allen, P.M. Brickell, C. Kinnon, G.A. Dunn, G.E. Jones, and A.J. Thrasher. 1998. Chemotaxis of macrophages is abolished in the Wiskott-Aldrich syndrome. *Br J Haematol* 101:659-665.
113. Binks, M., G.E. Jones, P.M. Brickell, C. Kinnon, D.R. Katz, and A.J. Thrasher. 1998. Intrinsic dendritic cell abnormalities in Wiskott-Aldrich syndrome. *Eur J Immunol* 28:3259-3267.
114. Linder, S., D. Nelson, M. Weiss, and M. Aepfelbacher. 1999. Wiskott-Aldrich syndrome protein regulates podosomes in primary human macrophages. *Proc Natl Acad Sci U S A* 96:9648-9653.
115. Tsuboi, S., and J. Meerloo. 2007. Wiskott-Aldrich syndrome protein is a key regulator of the phagocytic cup formation in macrophages. *J Biol Chem* 282:34194-34203.
116. de Noronha, S., S. Hardy, J. Sinclair, M.P. Blundell, J. Strid, O. Schulz, J. Zwirner, G.E. Jones, D.R. Katz, C. Kinnon, and A.J. Thrasher. 2005. Impaired dendritic-cell homing in vivo in the absence of Wiskott-Aldrich syndrome protein. *Blood* 105:1590-1597.
117. Bouma, G., S. Burns, and A.J. Thrasher. 2007. Impaired T-cell priming in vivo resulting from dysfunction of WASp-deficient dendritic cells. *Blood* 110:4278-4284.
118. Pulecio, J., E. Tagliani, A. Scholer, F. Prete, L. Fetler, O.R. Burrone, and F. Benvenuti. 2008. Expression of Wiskott-Aldrich syndrome protein in dendritic cells regulates synapse formation and activation of naive CD8+ T cells. *J Immunol* 181:1135-1142.
119. Pivniouk, V.I., S.B. Snapper, A. Kettner, H. Alenius, D. Laouini, H. Falet, J. Hartwig, F.W. Alt, and R.S. Geha. 2003. Impaired signaling via the high-affinity IgE receptor in Wiskott-Aldrich syndrome protein-deficient mast cells. *Int Immunol* 15:1431-1440.
120. Gross, B.S., J.I. Wilde, L. Quek, H. Chapel, D.L. Nelson, and S.P. Watson. 1999. Regulation and function of WASp in platelets by the collagen receptor, glycoprotein VI. *Blood* 94:4166-4176.
121. Hadden, J.W., B. Holmes-Gray, and R.A. Good. 1970. Platelet abnormality in Wiskott-Aldrich syndrome. *N Engl J Med* 283:876-877.

122. Bendelac, A., P.B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu Rev Immunol* 25:297-336.
123. Sumida, T., I. Takei, and M. Taniguchi. 1984. Activation of acceptor-suppressor hybridoma with antigen-specific suppressor T cell factor of two-chain type: requirement of the antigen- and the I-J-restricting specificity. *J Immunol* 133:1131-1136.
124. Sumida, T., and M. Taniguchi. 1985. Novel mechanisms of specific suppression of anti-hapten antibody response mediated by monoclonal anti-carrier antibody. *J Immunol* 134:3675-3681.
125. Imai, K., M. Kanno, H. Kimoto, K. Shigemoto, S. Yamamoto, and M. Taniguchi. 1986. Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. *Proc Natl Acad Sci U S A* 83:8708-8712.
126. Koseki, H., K. Imai, F. Nakayama, T. Sado, K. Moriwaki, and M. Taniguchi. 1990. Homogenous junctional sequence of the V14+ T-cell antigen receptor alpha chain expanded in unprimed mice. *Proc Natl Acad Sci U S A* 87:5248-5252.
127. Koseki, H., H. Asano, T. Inaba, N. Miyashita, K. Moriwaki, K.F. Lindahl, Y. Mizutani, K. Imai, and M. Taniguchi. 1991. Dominant expression of a distinctive V14+ T-cell antigen receptor alpha chain in mice. *Proc Natl Acad Sci U S A* 88:7518-7522.
128. Budd, R.C., G.C. Miescher, R.C. Howe, R.K. Lees, C. Bron, and H.R. MacDonald. 1987. Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes. *J Exp Med* 166:577-582.
129. Fowlkes, B.J., A.M. Kruisbeek, H. Ton-That, M.A. Weston, J.E. Coligan, R.H. Schwartz, and D.M. Pardoll. 1987. A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. *Nature* 329:251-254.
130. Porcelli, S., C.E. Yockey, M.B. Brenner, and S.P. Balk. 1993. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* 178:1-16.
131. Dellabona, P., E. Padovan, G. Casorati, M. Brockhaus, and A. Lanzavecchia. 1994. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med* 180:1171-1176.
132. Bendelac, A., and R.H. Schwartz. 1991. CD4+ and CD8+ T cells acquire specific lymphokine secretion potentials during thymic maturation. *Nature* 353:68-71.
133. Bendelac, A., P. Matzinger, R.A. Seder, W.E. Paul, and R.H. Schwartz. 1992. Activation events during thymic selection. *J Exp Med* 175:731-742.
134. Hayakawa, K., B.T. Lin, and R.R. Hardy. 1992. Murine thymic CD4+ T cell subsets: a subset (Thy0) that secretes diverse cytokines and overexpresses the V beta 8 T cell receptor gene family. *J Exp Med* 176:269-274.
135. Arase, H., N. Arase, K. Ogasawara, R.A. Good, and K. Onoe. 1992. An NK1.1+ CD4+8- single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor V beta family. *Proc Natl Acad Sci U S A* 89:6506-6510.

136. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med* 180:1097-1106.
137. Exley, M., J. Garcia, S.P. Balk, and S. Porcelli. 1997. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J Exp Med* 186:109-120.
138. Bendelac, A., N. Killeen, D.R. Littman, and R.H. Schwartz. 1994. A subset of CD4+ thymocytes selected by MHC class I molecules. *Science* 263:1774-1778.
139. Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz. 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268:863-865.
140. Bendelac, A. 1995. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med* 182:2091-2096.
141. Park, S.H., A. Weiss, K. Benlagha, T. Kyin, L. Teyton, and A. Bendelac. 2001. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. *J Exp Med* 193:893-904.
142. Cardell, S., S. Tangri, S. Chan, M. Kronenberg, C. Benoist, and D. Mathis. 1995. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* 182:993-1004.
143. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626-1629.
144. Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med* 188:1521-1528.
145. Mattner, J., K.L. Debord, N. Ismail, R.D. Goff, C. Cantu, 3rd, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P.B. Savage, and A. Bendelac. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434:525-529.
146. Kinjo, Y., D. Wu, G. Kim, G.W. Xing, M.A. Poles, D.D. Ho, M. Tsuji, K. Kawahara, C.H. Wong, and M. Kronenberg. 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434:520-525.
147. Sriram, V., W. Du, J. Gervay-Hague, and R.R. Brutkiewicz. 2005. Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. *Eur J Immunol* 35:1692-1701.
148. Zhou, D., J. Mattner, C. Cantu, 3rd, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y.P. Wu, T. Yamashita, S. Teneberg, D. Wang, R.L. Proia, S.B. Levery, P.B. Savage, L. Teyton, and A. Bendelac. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306:1786-1789.
149. Matsuda, J.L., O.V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192:741-754.
150. Brigl, M., and M.B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 22:817-890.

151. Skold, M., and S. Cardell. 2000. Differential regulation of Ly49 expression on CD4+ and CD4-CD8- (double negative) NK1.1+ T cells. *Eur J Immunol* 30:2488-2496.
152. Godfrey, D.I., and S.P. Berzins. 2007. Control points in NKT-cell development. *Nat Rev Immunol* 7:505-518.
153. Bendelac, A., M.N. Rivera, S.H. Park, and J.H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 15:535-562.
154. D'Andrea, A., D. Goux, C. De Lalla, Y. Koezuka, D. Montagna, A. Moretta, P. Dellabona, G. Casorati, and S. Abrignani. 2000. Neonatal invariant Valpha24+ NKT lymphocytes are activated memory cells. *Eur J Immunol* 30:1544-1550.
155. van Der Vliet, H.J., N. Nishi, T.D. de Gruijl, B.M. von Blomberg, A.J. van den Eertwegh, H.M. Pinedo, G. Giaccone, and R.J. Scheper. 2000. Human natural killer T cells acquire a memory-activated phenotype before birth. *Blood* 95:2440-2442.
156. Park, S.H., K. Benlagha, D. Lee, E. Balish, and A. Bendelac. 2000. Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice. *Eur J Immunol* 30:620-625.
157. Hammond, K.J., S.B. Pelikan, N.Y. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M.J. Smyth, I.R. van Driel, R. Scollay, A.G. Baxter, and D.I. Godfrey. 1999. NKT cells are phenotypically and functionally diverse. *Eur J Immunol* 29:3768-3781.
158. Gumperz, J.E., S. Miyake, T. Yamamura, and M.B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195:625-636.
159. Hammond, K.J., D.G. Pellicci, L.D. Poulton, O.V. Naidenko, A.A. Scalzo, A.G. Baxter, and D.I. Godfrey. 2001. CD1d-restricted NKT cells: an interstrain comparison. *J Immunol* 167:1164-1173.
160. Kim, C.H., B. Johnston, and E.C. Butcher. 2002. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. *Blood* 100:11-16.
161. Thomas, S.Y., R. Hou, J.E. Boyson, T.K. Means, C. Hess, D.P. Olson, J.L. Strominger, M.B. Brenner, J.E. Gumperz, S.B. Wilson, and A.D. Luster. 2003. CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. *J Immunol* 171:2571-2580.
162. Johnston, B., C.H. Kim, D. Soler, M. Emoto, and E.C. Butcher. 2003. Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. *J Immunol* 171:2960-2969.
163. Geissmann, F., T.O. Cameron, S. Sidobre, N. Manlongat, M. Kronenberg, M.J. Briskin, M.L. Dustin, and D.R. Littman. 2005. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol* 3:e113.
164. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NK T cell lineage. *Science* 296:553-555.
165. Pellicci, D.G., K.J. Hammond, A.P. Uldrich, A.G. Baxter, M.J. Smyth, and D.I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J Exp Med* 195:835-844.

166. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* 7:529-534.
167. Morita, M., K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, and H. Fukushima. 1995. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* 38:2176-2187.
168. Kinjo, Y., E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M.R. Benhnia, D.M. Zajonc, G. Ben-Menachem, G.D. Ainge, G.F. Painter, A. Khurana, K. Hoebe, S.M. Behar, B. Beutler, I.A. Wilson, M. Tsuji, T.J. Sellati, C.H. Wong, and M. Kronenberg. 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* 7:978-986.
169. Kumar, H., A. Belperron, S.W. Barthold, and L.K. Bockenstedt. 2000. Cutting edge: CD1d deficiency impairs murine host defense against the spirochete, Borrelia burgdorferi. *J Immunol* 165:4797-4801.
170. Park, S.H., J.H. Roark, and A. Bendelac. 1998. Tissue-specific recognition of mouse CD1 molecules. *J Immunol* 160:3128-3134.
171. Brigl, M., L. Bry, S.C. Kent, J.E. Gumperz, and M.B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* 4:1230-1237.
172. Xia, C., Q. Yao, J. Schumann, E. Rossy, W. Chen, L. Zhu, W. Zhang, G. De Libero, and P.G. Wang. 2006. Synthesis and biological evaluation of alpha-galactosylceramide (KRN7000) and isoglobotrihexosylceramide (iGb3). *Bioorg Med Chem Lett* 16:2195-2199.
173. Porubsky, S., A.O. Speak, B. Luckow, V. Cerundolo, F.M. Platt, and H.J. Grone. 2007. Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. *Proc Natl Acad Sci U S A* 104:5977-5982.
174. Roark, J.H., S.H. Park, J. Jayawardena, U. Kavita, M. Shannon, and A. Bendelac. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160:3121-3127.
175. Brossay, L., D. Jullien, S. Cardell, B.C. Sydora, N. Burdin, R.L. Modlin, and M. Kronenberg. 1997. Mouse CD1 is mainly expressed on hemopoietic-derived cells. *J Immunol* 159:1216-1224.
176. Wei, D.G., H. Lee, S.H. Park, L. Beaudoin, L. Teyton, A. Lehuen, and A. Bendelac. 2005. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J Exp Med* 202:239-248.
177. Kang, S.J., and P. Cresswell. 2002. Calnexin, calreticulin, and ERp57 cooperate in disulfide bond formation in human CD1d heavy chain. *J Biol Chem* 277:44838-44844.
178. Paduraru, C., L. Spiridon, W. Yuan, G. Bricard, X. Valencia, S.A. Porcelli, P.A. Illarionov, G.S. Besra, S.M. Petrescu, A.J. Petrescu, and P. Cresswell. 2006. An N-linked glycan modulates the interaction between the CD1d heavy chain and beta 2-microglobulin. *J Biol Chem* 281:40369-40378.
179. De Silva, A.D., J.J. Park, N. Matsuki, A.K. Stanic, R.R. Brutkiewicz, M.E. Medof, and S. Joyce. 2002. Lipid protein interactions: the assembly of CD1d1 with cellular phospholipids occurs in the endoplasmic reticulum. *J Immunol* 168:723-733.

180. Jayawardena-Wolf, J., K. Benlagha, Y.H. Chiu, R. Mehr, and A. Bendelac. 2001. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* 15:897-908.
181. Roberts, T.J., V. Sriram, P.M. Spence, M. Gui, K. Hayakawa, I. Bacik, J.R. Bennink, J.W. Yewdell, and R.R. Brutkiewicz. 2002. Recycling CD1d1 molecules present endogenous antigens processed in an endocytic compartment to NKT cells. *J Immunol* 168:5409-5414.
182. Lawton, A.P., T.I. Prigozy, L. Brossay, B. Pei, A. Khurana, D. Martin, T. Zhu, K. Spate, M. Ozga, S. Honing, O. Bakke, and M. Kronenberg. 2005. The mouse CD1d cytoplasmic tail mediates CD1d trafficking and antigen presentation by adaptor protein 3-dependent and -independent mechanisms. *J Immunol* 174:3179-3186.
183. Cernadas, M., M. Sugita, N. van der Wel, X. Cao, J.E. Gumperz, S. Maltsev, G.S. Besra, S.M. Behar, P.J. Peters, and M.B. Brenner. 2003. Lysosomal localization of murine CD1d mediated by AP-3 is necessary for NK T cell development. *J Immunol* 171:4149-4155.
184. Sugita, M., X. Cao, G.F. Watts, R.A. Rogers, J.S. Bonifacino, and M.B. Brenner. 2002. Failure of trafficking and antigen presentation by CD1 in AP-3-deficient cells. *Immunity* 16:697-706.
185. Chiu, Y.H., S.H. Park, K. Benlagha, C. Forestier, J. Jayawardena-Wolf, P.B. Savage, L. Teyton, and A. Bendelac. 2002. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail-truncated CD1d. *Nat Immunol* 3:55-60.
186. Elewaut, D., A.P. Lawton, N.A. Nagarajan, E. Maverakis, A. Khurana, S. Honing, C.A. Benedict, E. Sercarz, O. Bakke, M. Kronenberg, and T.I. Prigozy. 2003. The adaptor protein AP-3 is required for CD1d-mediated antigen presentation of glycosphingolipids and development of Valpha14i NKT cells. *J Exp Med* 198:1133-1146.
187. Silk, J.D., M. Salio, J. Brown, E.Y. Jones, and V. Cerundolo. 2008. Structural and functional aspects of lipid binding by CD1 molecules. *Annu Rev Cell Dev Biol* 24:369-395.
188. Zhou, D., C. Cantu, 3rd, Y. Sagiv, N. Schrantz, A.B. Kulkarni, X. Qi, D.J. Mahuran, C.R. Morales, G.A. Grabowski, K. Benlagha, P. Savage, A. Bendelac, and L. Teyton. 2004. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science* 303:523-527.
189. Kang, S.J., and P. Cresswell. 2004. Saposins facilitate CD1d-restricted presentation of an exogenous lipid antigen to T cells. *Nat Immunol* 5:175-181.
190. Schrantz, N., Y. Sagiv, Y. Liu, P.B. Savage, A. Bendelac, and L. Teyton. 2007. The Niemann-Pick type C2 protein loads isoglobotrihexosylceramide onto CD1d molecules and contributes to the thymic selection of NKT cells. *J Exp Med* 204:841-852.
191. Im, J.S., P. Arora, G. Bricard, A. Molano, M.M. Venkataswamy, I. Baine, E.S. Jerud, M.F. Goldberg, A. Baena, K.O. Yu, R.M. Ndonye, A.R. Howell, W. Yuan, P. Cresswell, Y.T. Chang, P.A. Illarionov, G.S. Besra, and S.A. Porcelli. 2009. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. *Immunity* 30:888-898.
192. Makino, Y., R. Kanno, H. Koseki, and M. Taniguchi. 1996. Development of Valpha4+ NK T cells in the early stages of embryogenesis. *Proc Natl Acad Sci U S A* 93:6516-6520.

193. Sato, H., T. Nakayama, Y. Tanaka, M. Yamashita, Y. Shibata, E. Kondo, Y. Saito, and M. Taniguchi. 1999. Induction of differentiation of pre-NKT cells to mature Valpha14 NKT cells by granulocyte/macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A* 96:7439-7444.
194. Levitsky, H.I., P.T. Golumbek, and D.M. Pardoll. 1991. The fate of CD4-8- T cell receptor-alpha beta+ thymocytes. *J Immunol* 146:1113-1117.
195. Tilloy, F., J.P. Di Santo, A. Bendelac, and O. Lantz. 1999. Thymic dependence of invariant V alpha 14+ natural killer-T cell development. *Eur J Immunol* 29:3313-3318.
196. Coles, M.C., and D.H. Raulet. 2000. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol* 164:2412-2418.
197. Hammond, K., W. Cain, I. van Driel, and D. Godfrey. 1998. Three day neonatal thymectomy selectively depletes NK1.1+ T cells. *Int Immunol* 10:1491-1499.
198. Benlagha, K., D.G. Wei, J. Veiga, L. Teyton, and A. Bendelac. 2005. Characterization of the early stages of thymic NKT cell development. *J Exp Med* 202:485-492.
199. Gadue, P., and P.L. Stein. 2002. NK T cell precursors exhibit differential cytokine regulation and require Itk for efficient maturation. *J Immunol* 169:2397-2406.
200. Gapin, L., J.L. Matsuda, C.D. Surh, and M. Kronenberg. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat Immunol* 2:971-978.
201. Shimamura, M., T. Ohteki, U. Beutner, and H.R. MacDonald. 1997. Lack of directed V alpha 14-J alpha 281 rearrangements in NK1+ T cells. *Eur J Immunol* 27:1576-1579.
202. Egawa, T., G. Eberl, I. Taniuchi, K. Benlagha, F. Geissmann, L. Hennighausen, A. Bendelac, and D.R. Littman. 2005. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* 22:705-716.
203. Dao, T., D. Guo, A. Ploss, A. Stolzer, C. Saylor, T.E. Boursalian, J.S. Im, and D.B. Sant'Angelo. 2004. Development of CD1d-restricted NKT cells in the mouse thymus. *Eur J Immunol* 34:3542-3552.
204. Wei, D.G., S.A. Curran, P.B. Savage, L. Teyton, and A. Bendelac. 2006. Mechanisms imposing the Vbeta bias of Valpha14 natural killer T cells and consequences for microbial glycolipid recognition. *J Exp Med* 203:1197-1207.
205. Schumann, J., R.B. Voyle, B.Y. Wei, and H.R. MacDonald. 2003. Cutting edge: influence of the TCR V beta domain on the avidity of CD1d:alpha-galactosylceramide binding by invariant V alpha 14 NKT cells. *J Immunol* 170:5815-5819.
206. Schumann, J., M.P. Mycko, P. Dellabona, G. Casorati, and H.R. MacDonald. 2006. Cutting edge: influence of the TCR Vbeta domain on the selection of semi-invariant NKT cells by endogenous ligands. *J Immunol* 176:2064-2068.
207. Chun, T., M.J. Page, L. Gapin, J.L. Matsuda, H. Xu, H. Nguyen, H.S. Kang, A.K. Stanic, S. Joyce, W.A. Koltun, M.J. Chorney, M. Kronenberg, and C.R. Wang. 2003. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med* 197:907-918.
208. Pellicci, D.G., A.P. Uldrich, K. Kyparissoudis, N.Y. Crowe, A.G. Brooks, K.J. Hammond, S. Sidobre, M. Kronenberg, M.J. Smyth, and D.I. Godfrey. 2003.

Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *Eur J Immunol* 33:1816-1823.
209. McNab, F.W., S.P. Berzins, D.G. Pellicci, K. Kyparissoudis, K. Field, M.J. Smyth, and D.I. Godfrey. 2005. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol* 175:3762-3768.
210. Berzins, S.P., F.W. McNab, C.M. Jones, M.J. Smyth, and D.I. Godfrey. 2006. Long-term retention of mature NK1.1+ NKT cells in the thymus. *J Immunol* 176:4059-4065.
211. McNab, F.W., D.G. Pellicci, K. Field, G. Besra, M.J. Smyth, D.I. Godfrey, and S.P. Berzins. 2007. Peripheral NK1.1 NKT cells are mature and functionally distinct from their thymic counterparts. *J Immunol* 179:6630-6637.
212. Baev, D.V., X.H. Peng, L. Song, J.R. Barnhart, G.M. Crooks, K.I. Weinberg, and L.S. Metelitsa. 2004. Distinct homeostatic requirements of CD4+ and CD4- subsets of Valpha24-invariant natural killer T cells in humans. *Blood* 104:4150-4156.
213. Berzins, S.P., A.D. Cochrane, D.G. Pellicci, M.J. Smyth, and D.I. Godfrey. 2005. Limited correlation between human thymus and blood NKT cell content revealed by an ontogeny study of paired tissue samples. *Eur J Immunol* 35:1399-1407.
214. Stanic, A.K., J.S. Bezbradica, J.J. Park, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase C theta and NF-kappa B. *J Immunol* 172:4667-4671.
215. Stanic, A.K., J.S. Bezbradica, J.J. Park, N. Matsuki, A.L. Mora, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *J Immunol* 172:2265-2273.
216. Elewaut, D., R.B. Shaikh, K.J. Hammond, H. De Winter, A.J. Leishman, S. Sidobre, O. Turovskaya, T.I. Prigozy, L. Ma, T.A. Banks, D. Lo, C.F. Ware, H. Cheroutre, and M. Kronenberg. 2003. NIK-dependent RelB activation defines a unique signaling pathway for the development of V alpha 14i NKT cells. *J Exp Med* 197:1623-1633.
217. Sivakumar, V., K.J. Hammond, N. Howells, K. Pfeffer, and F. Weih. 2003. Differential requirement for Rel/nuclear factor kappa B family members in natural killer T cell development. *J Exp Med* 197:1613-1621.
218. Sivakumar, V. 2008. Rel/NF-kB family member RelA regulates NK1.1- to NK1.1+ transition as well as IL-15-induced expansion of NKT cells. *European Journal of Immunology* 38:3508-3519.
219. Lazarevic, V., A.J. Zullo, M.N. Schweitzer, T.L. Staton, E.M. Gallo, G.R. Crabtree, and L.H. Glimcher. 2009. The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells. *Nat Immunol* 10:306-313.
220. Zullo, A.J., K. Benlagha, A. Bendelac, and E.J. Taparowsky. 2007. Sensitivity of NK1.1-negative NKT cells to transgenic BATF defines a role for activator protein-1 in the expansion and maturation of immature NKT cells in the thymus. *J Immunol* 178:58-66.
221. Townsend, M.J., A.S. Weinmann, J.L. Matsuda, R. Salomon, P.J. Farnham, C.A. Biron, L. Gapin, and L.H. Glimcher. 2004. T-bet regulates the terminal

maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20:477-494.
222. Matsuda, J.L., Q. Zhang, R. Ndonye, S.K. Richardson, A.R. Howell, and L. Gapin. 2006. T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood* 107:2797-2805.
223. Felices, M., and L.J. Berg. 2008. The Tec kinases Itk and Rlk regulate NKT cell maturation, cytokine production, and survival. *J Immunol* 180:3007-3018.
224. Williams, J.A., J.M. Lumsden, X. Yu, L. Feigenbaum, J. Zhang, S.M. Steinberg, and R.J. Hodes. 2008. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. *J Immunol* 181:907-917.
225. Zheng, X., H. Zhang, L. Yin, C.R. Wang, Y. Liu, and P. Zheng. 2008. Modulation of NKT cell development by B7-CD28 interaction: an expanding horizon for costimulation. *PLoS One* 3:e2703.
226. Akbari, O., P. Stock, E.H. Meyer, G.J. Freeman, A.H. Sharpe, D.T. Umetsu, and R.H. DeKruyff. 2008. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol* 180:5448-5456.
227. Pasquier, B., L. Yin, M.C. Fondaneche, F. Relouzat, C. Bloch-Queyrat, N. Lambert, A. Fischer, G. de Saint-Basile, and S. Latour. 2005. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med* 201:695-701.
228. Nichols, K.E., J. Hom, S.Y. Gong, A. Ganguly, C.S. Ma, J.L. Cannons, S.G. Tangye, P.L. Schwartzberg, G.A. Koretzky, and P.L. Stein. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* 11:340-345.
229. Griewank, K., C. Borowski, S. Rietdijk, N. Wang, A. Julien, D.G. Wei, A.A. Mamchak, C. Terhorst, and A. Bendelac. 2007. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27:751-762.
230. Eberl, G., B. Lowin-Kropf, and H.R. MacDonald. 1999. Cutting edge: NKT cell development is selectively impaired in Fyn- deficient mice. *J Immunol* 163:4091-4094.
231. Gadue, P., N. Morton, and P.L. Stein. 1999. The Src family tyrosine kinase Fyn regulates natural killer T cell development. *J Exp Med* 190:1189-1196.
232. Matsuda, J.L., L. Gapin, S. Sidobre, W.C. Kieper, J.T. Tan, R. Ceredig, C.D. Surh, and M. Kronenberg. 2002. Homeostasis of V alpha 14i NKT cells. *Nat Immunol* 3:966-974.
233. Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, M. Taniguchi, and T. Nishimura. 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 189:1121-1128.
234. Yoshimoto, T., and W.E. Paul. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med* 179:1285-1295.
235. Hayakawa, Y., K. Takeda, H. Yagita, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J Immunol* 166:6012-6018.

236. Vincent, M.S., D.S. Leslie, J.E. Gumperz, X. Xiong, E.P. Grant, and M.B. Brenner. 2002. CD1-dependent dendritic cell instruction. *Nat Immunol* 3:1163-1168.
237. Tomura, M., W.G. Yu, H.J. Ahn, M. Yamashita, Y.F. Yang, S. Ono, T. Hamaoka, T. Kawano, M. Taniguchi, Y. Koezuka, and H. Fujiwara. 1999. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J Immunol* 163:93-101.
238. Kawamura, T., K. Takeda, S.K. Mendiratta, H. Kawamura, L. Van Kaer, H. Yagita, T. Abo, and K. Okumura. 1998. Critical role of NK1+ T cells in IL-12-induced immune responses in vivo. *J Immunol* 160:16-19.
239. Yang, Y.F., M. Tomura, S. Ono, T. Hamaoka, and H. Fujiwara. 2000. Requirement for IFN-gamma in IL-12 production induced by collaboration between v(alpha)14(+) NKT cells and antigen-presenting cells. *Int Immunol* 12:1669-1675.
240. Tupin, E., Y. Kinjo, and M. Kronenberg. 2007. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 5:405-417.
241. Mallevaey, T., J.P. Zanetta, C. Faveeuw, J. Fontaine, E. Maes, F. Platt, M. Capron, M.L. de-Moraes, and F. Trottein. 2006. Activation of invariant NKT cells by the helminth parasite schistosoma mansoni. *J Immunol* 176:2476-2485.
242. Nagarajan, N.A., and M. Kronenberg. 2007. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J Immunol* 178:2706-2713.
243. Arase, H., N. Arase, K. Nakagawa, R.A. Good, and K. Onoe. 1993. NK1.1+ CD4+ CD8- thymocytes with specific lymphokine secretion. *Eur J Immunol* 23:307-310.
244. Matsuda, J.L., L. Gapin, J.L. Baron, S. Sidobre, D.B. Stetson, M. Mohrs, R.M. Locksley, and M. Kronenberg. 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc Natl Acad Sci U S A* 100:8395-8400.
245. Stetson, D.B., M. Mohrs, R.L. Reinhardt, J.L. Baron, Z.E. Wang, L. Gapin, M. Kronenberg, and R.M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* 198:1069-1076.
246. Van Kaer, L. 2005. alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat Rev Immunol* 5:31-42.
247. Carnaud, C., D. Lee, O. Donnars, S.H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* 163:4647-4650.
248. Eberl, G., and H.R. MacDonald. 2000. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur J Immunol* 30:985-992.
249. Eberl, G., P. Brawand, and H.R. MacDonald. 2000. Selective bystander proliferation of memory CD4+ and CD8+ T cells upon NK T or T cell activation. *J Immunol* 165:4305-4311.
250. Metelitsa, L.S., O.V. Naidenko, A. Kant, H.W. Wu, M.J. Loza, B. Perussia, M. Kronenberg, and R.C. Seeger. 2001. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* 167:3114-3122.

251. Nieuwenhuis, E.E., T. Matsumoto, M. Exley, R.A. Schleipman, J. Glickman, D.T. Bailey, N. Corazza, S.P. Colgan, A.B. Onderdonk, and R.S. Blumberg. 2002. CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung. *Nat Med* 8:588-593.
252. Galli, G., S. Nuti, S. Tavarini, L. Galli-Stampino, C. De Lalla, G. Casorati, P. Dellabona, and S. Abrignani. 2003. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med* 197:1051-1057.
253. Galli, G., P. Pittoni, E. Tonti, C. Malzone, Y. Uematsu, M. Tortoli, D. Maione, G. Volpini, O. Finco, S. Nuti, S. Tavarini, P. Dellabona, R. Rappuoli, G. Casorati, and S. Abrignani. 2007. Invariant NKT cells sustain specific B cell responses and memory. *Proc Natl Acad Sci U S A* 104:3984-3989.
254. Wang, Z.Y., S. Kusam, V. Munugalavadla, R. Kapur, R.R. Brutkiewicz, and A.L. Dent. 2006. Regulation of Th2 cytokine expression in NKT cells: unconventional use of Stat6, GATA-3, and NFAT2. *J Immunol* 176:880-888.
255. Kim, P.J., S.Y. Pai, M. Brigl, G.S. Besra, J. Gumperz, and I.C. Ho. 2006. GATA-3 regulates the development and function of invariant NKT cells. *J Immunol* 177:6650-6659.
256. Kawakami, K., N. Yamamoto, Y. Kinjo, K. Miyagi, C. Nakasone, K. Uezu, T. Kinjo, T. Nakayama, M. Taniguchi, and A. Saito. 2003. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. *Eur J Immunol* 33:3322-3330.
257. Mattner, J., N. Donhauser, G. Werner-Felmayer, and C. Bogdan. 2006. NKT cells mediate organ-specific resistance against Leishmania major infection. *Microbes Infect* 8:354-362.
258. Grubor-Bauk, B., A. Simmons, G. Mayrhofer, and P.G. Speck. 2003. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol* 170:1430-1434.
259. Cornish, A.L., R. Keating, K. Kyparissoudis, M.J. Smyth, F.R. Carbone, and D.I. Godfrey. 2006. NKT cells are not critical for HSV-1 disease resolution. *Immunol Cell Biol* 84:13-19.
260. Ashkar, A.A., and K.L. Rosenthal. 2003. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. *J Virol* 77:10168-10171.
261. Bilenki, L., S. Wang, J. Yang, Y. Fan, A.G. Joyee, and X. Yang. 2005. NK T cell activation promotes Chlamydia trachomatis infection in vivo. *J Immunol* 175:3197-3206.
262. Smyth, M.J., E. Cretney, K. Takeda, R.H. Wiltrout, L.M. Sedger, N. Kayagaki, H. Yagita, and K. Okumura. 2001. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med* 193:661-670.
263. Nakagawa, R., K. Motoki, H. Ueno, R. Iijima, H. Nakamura, E. Kobayashi, A. Shimosaka, and Y. Koezuka. 1998. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res* 58:1202-1207.
264. Smyth, M.J., N.Y. Crowe, D.G. Pellicci, K. Kyparissoudis, J.M. Kelly, K. Takeda, H. Yagita, and D.I. Godfrey. 2002. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* 99:1259-1266.

265. Hayakawa, Y., K. Takeda, H. Yagita, S. Kakuta, Y. Iwakura, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol* 31:1720-1727.
266. Nakagawa, R., I. Nagafune, Y. Tazunoki, H. Ehara, H. Tomura, R. Iijima, K. Motoki, M. Kamishohara, and S. Seki. 2001. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. *J Immunol* 166:6578-6584.
267. Smyth, M.J., K.Y. Thia, S.E. Street, E. Cretney, J.A. Trapani, M. Taniguchi, T. Kawano, S.B. Pelikan, N.Y. Crowe, and D.I. Godfrey. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* 191:661-668.
268. Smyth, M.J., N.Y. Crowe, and D.I. Godfrey. 2001. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* 13:459-463.
269. Crowe, N.Y., M.J. Smyth, and D.I. Godfrey. 2002. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* 196:119-127.
270. Tahir, S.M., O. Cheng, A. Shaulov, Y. Koezuka, G.J. Bubley, S.B. Wilson, S.P. Balk, and M.A. Exley. 2001. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* 167:4046-4050.
271. Fujii, S., K. Shimizu, V. Klimek, M.D. Geller, S.D. Nimer, and M.V. Dhodapkar. 2003. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br J Haematol* 122:617-622.
272. Hong, S., M.T. Wilson, I. Serizawa, L. Wu, N. Singh, O.V. Naidenko, T. Miura, T. Haba, D.C. Scherer, J. Wei, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 2001. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* 7:1052-1056.
273. Wang, B., Y.B. Geng, and C.R. Wang. 2001. CD1-restricted NK T cells protect nonobese diabetic mice from developing diabetes. *J Exp Med* 194:313-320.
274. Shi, F.D., M. Flodstrom, B. Balasa, S.H. Kim, K. Van Gunst, J.L. Strominger, S.B. Wilson, and N. Sarvetnick. 2001. Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse. *Proc Natl Acad Sci U S A* 98:6777-6782.
275. Naumov, Y.N., K.S. Bahjat, R. Gausling, R. Abraham, M.A. Exley, Y. Koezuka, S.B. Balk, J.L. Strominger, M. Clare-Salzer, and S.B. Wilson. 2001. Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. *Proc Natl Acad Sci U S A* 98:13838-13843.
276. Hammond, K.J., L.D. Poulton, L.J. Palmisano, P.A. Silveira, D.I. Godfrey, and A.G. Baxter. 1998. alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* 187:1047-1056.
277. Lehuen, A., O. Lantz, L. Beaudoin, V. Laloux, C. Carnaud, A. Bendelac, J.F. Bach, and R.C. Monteiro. 1998. Overexpression of natural killer T cells

protects Valpha14- Jalpha281 transgenic nonobese diabetic mice against diabetes. *J Exp Med* 188:1831-1839.
278. Sharif, S., G.A. Arreaza, P. Zucker, Q.S. Mi, J. Sondhi, O.V. Naidenko, M. Kronenberg, Y. Koezuka, T.L. Delovitch, J.M. Gombert, M. Leite-De-Moraes, C. Gouarin, R. Zhu, A. Hameg, T. Nakayama, M. Taniguchi, F. Lepault, A. Lehuen, J.F. Bach, and A. Herbelin. 2001. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat Med* 7:1057-1062.
279. Hammond, K.J., and M. Kronenberg. 2003. Natural killer T cells: natural or unnatural regulators of autoimmunity? *Curr Opin Immunol* 15:683-689.
280. Jahng, A.W., I. Maricic, B. Pedersen, N. Burdin, O. Naidenko, M. Kronenberg, Y. Koezuka, and V. Kumar. 2001. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 194:1789-1799.
281. Singh, A.K., M.T. Wilson, S. Hong, D. Olivares-Villagomez, C. Du, A.K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194:1801-1811.
282. Pal, E., T. Tabira, T. Kawano, M. Taniguchi, S. Miyake, and T. Yamamura. 2001. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of V alpha 14 NK T cells. *J Immunol* 166:662-668.
283. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413:531-534.
284. Zeng, D., Y. Liu, S. Sidobre, M. Kronenberg, and S. Strober. 2003. Activation of natural killer T cells in NZB/W mice induces Th1-type immune responses exacerbating lupus. *J Clin Invest* 112:1211-1222.
285. Forestier, C., A. Molano, J.S. Im, Y. Dutronc, B. Diamond, A. Davidson, P.A. Illarionov, G.S. Besra, and S.A. Porcelli. 2005. Expansion and hyperactivity of CD1d-restricted NKT cells during the progression of systemic lupus erythematosus in (New Zealand Black x New Zealand White)F1 mice. *J Immunol* 175:763-770.
286. Chan, O.T., V. Paliwal, J.M. McNiff, S.H. Park, A. Bendelac, and M.J. Shlomchik. 2001. Deficiency in beta(2)-microglobulin, but not CD1, accelerates spontaneous lupus skin disease while inhibiting nephritis in MRL-Fas(lpr) nice: an example of disease regulation at the organ level. *J Immunol* 167:2985-2990.
287. Yang, J.Q., T. Chun, H. Liu, S. Hong, H. Bui, L. Van Kaer, C.R. Wang, and R.R. Singh. 2004. CD1d deficiency exacerbates inflammatory dermatitis in MRL-lpr/lpr mice. *Eur J Immunol* 34:1723-1732.
288. Yang, J.Q., A.K. Singh, M.T. Wilson, M. Satoh, A.K. Stanic, J.J. Park, S. Hong, S.D. Gadola, A. Mizutani, S.R. Kakumanu, W.H. Reeves, V. Cerundolo, S. Joyce, L. Van Kaer, and R.R. Singh. 2003. Immunoregulatory role of CD1d in the hydrocarbon oil-induced model of lupus nephritis. *J Immunol* 171:2142-2153.
289. Singh, A.K., J.Q. Yang, V.V. Parekh, J. Wei, C.R. Wang, S. Joyce, R.R. Singh, and L. Van Kaer. 2005. The natural killer T cell ligand alpha-galactosylceramide prevents or promotes pristane-induced lupus in mice. *Eur J Immunol* 35:1143-1154.

290. Latour, S. 2007. Natural killer T cells and X-linked lymphoproliferative syndrome. *Curr Opin Allergy Clin Immunol* 7:510-514.
291. Rigaud, S., M.C. Fondaneche, N. Lambert, B. Pasquier, V. Mateo, P. Soulas, L. Galicier, F. Le Deist, F. Rieux-Laucat, P. Revy, A. Fischer, G. de Saint Basile, and S. Latour. 2006. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature* 444:110-114.
292. Matangkasombut, P., M. Pichavant, D.E. Saez, S. Giliani, E. Mazzolari, A. Finocchi, A. Villa, C. Sobacchi, P. Cortes, D.T. Umetsu, and L.D. Notarangelo. 2008. Lack of iNKT cells in patients with combined immune deficiency due to hypomorphic RAG mutations. *Blood* 111:271-274.
293. Rogers, P.R., A. Matsumoto, O. Naidenko, M. Kronenberg, T. Mikayama, and S. Kato. 2004. Expansion of human Valpha24+ NKT cells by repeated stimulation with KRN7000. *J Immunol Methods* 285:197-214.
294. Karadimitris, A., S. Gadola, M. Altamirano, D. Brown, A. Woolfson, P. Klenerman, J.L. Chen, Y. Koezuka, I.A. Roberts, D.A. Price, G. Dusheiko, C. Milstein, A. Fersht, L. Luzzatto, and V. Cerundolo. 2001. Human CD1d-glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci U S A* 98:3294-3298.
295. Wilson, M.T., C. Johansson, D. Olivares-Villagomez, A.K. Singh, A.K. Stanic, C.R. Wang, S. Joyce, M.J. Wick, and L. Van Kaer. 2003. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc Natl Acad Sci U S A* 100:10913-10918.
296. Astrakhan, A., H.D. Ochs, and D.J. Rawlings. 2009. Wiskott-Aldrich syndrome protein is required for homeostasis and function of invariant NKT cells. *J Immunol* 182:7370-7380.
297. Allende, M.L., D. Zhou, D.N. Kalkofen, S. Benhamed, G. Tuymetova, C. Borowski, A. Bendelac, and R.L. Proia. 2008. S1P1 receptor expression regulates emergence of NKT cells in peripheral tissues. *Faseb J* 22:307-315.
298. Meyer-Bahlburg, A., S. Becker-Herman, S. Humblet-Baron, S. Khim, M. Weber, G. Bouma, A.J. Thrasher, F.D. Batista, and D.J. Rawlings. 2008. Wiskott-Aldrich syndrome protein deficiency in B cells results in impaired peripheral homeostasis. *Blood* 112:4158-4169.
299. Westerberg, L.S., M.A. de la Fuente, F. Wermeling, H.D. Ochs, M.C. Karlsson, S.B. Snapper, and L.D. Notarangelo. 2008. WASP confers selective advantage for specific hematopoietic cell populations and serves a unique role in marginal zone B-cell homeostasis and function. *Blood* 112:4139-4147.
300. Shiow, L.R., D.B. Rosen, N. Brdickova, Y. Xu, J. An, L.L. Lanier, J.G. Cyster, and M. Matloubian. 2006. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 440:540-544.
301. Emoto, M., H.W. Mittrucker, R. Schmits, T.W. Mak, and S.H. Kaufmann. 1999. Critical role of leukocyte function-associated antigen-1 in liver accumulation of CD4+NKT cells. *J Immunol* 162:5094-5098.

8. PUBLISHED PAPERS

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The Wiskott-Aldrich syndrome protein is required for iNKT cell maturation and function

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The Wiskott-Aldrich syndrome (WAS) protein (WASp) is a regulator of actin cytoskeleton in hematopoietic cells. Mutations of the WASp gene cause WAS. Although WASp is involved in various immune cell functions, its role in invariant natural killer T (iNKT) cells has never been investigated. Defects of iNKT cells could indeed contribute to several WAS features, such as recurrent infections and high tumor incidence. We found a profound reduction of circulating iNKT cells in WAS patients, directly correlating with the severity of clinical phenotype. To better characterize iNKT cell defect in the absence of WASp, we analyzed *was*$^{-/-}$ mice. iNKT cell numbers were significantly reduced in the thymus and periphery of *was*$^{-/-}$ mice as compared with wild-type controls. Moreover analysis of *was*$^{-/-}$ iNKT cell maturation revealed a complete arrest at the CD44^{+} NK1.1^{-} intermediate stage. Notably, generation of BM chimeras demonstrated a *was*$^{-/-}$ iNKT cell-autonomous developmental defect. *was*$^{-/-}$ iNKT cells were also functionally impaired, as suggested by the reduced secretion of interleukin 4 and interferon γ upon in vivo activation. Altogether, these results demonstrate the relevance of WASp in integrating signals critical for development and functional differentiation of iNKT cells and suggest that defects in these cells may play a role in WAS pathology.

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Invariant natural killer T (iNKT) cells constitute a peculiar T lymphocyte subset, which is characterized by the coexpression of NK markers and an invariant TCR-α chain (Vα14Jα18 in mouse or Vα24Jα18 in human), which pairs with a restricted number of TCR-β chains (Vβ8, Vβ7, and Vβ2 in mice and Vβ11 in human). iNKT cells recognize glycolipid antigens, such as α-galactosylceramide (α-GalCer), presented in the context of CD1d molecules (1). iNKT cells develop in the thymus from CD4^{+} CD8^{+} double-positive (DP) cells that have randomly rearranged the semiinvariant TCR and are

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positively selected by recognition of CD1d molecules on DP thymocytes. After positive selection, the most immature iNKT cells (stage 1, CD44$^-$ NK1.1$^-$) first differentiate into CD44$^+$ NK1.1$^-$ (stage 2) and then are either exported into the periphery or remain in the thymus. In both compartments, iNKT cells complete their maturation, becoming CD44$^+$ NK1.1$^+$ mature cells (stage 3) (2). This differentiation program requires signaling molecules, adapters, and transcription factors that selectively control the development of iNKT and not of mainstream T cells (2).

Mature iNKT cells are strong immunoregulatory elements because they promptly produce a wide range of cytokines upon TCR triggering (1). iNKT cells are indeed involved in the control of pathogen infection and cancer immunosurveillance (3, 4) and play a protective role in many autoimmune diseases, although in some autoimmune mouse models they can exert a detrimental activity (5).

Interestingly, a complete lack of iNKT cells was found in the X-linked lymphoproliferative disease (XLP) (6), a primary immunodeficiency which is caused by mutations in SAP and XIAP genes and characterized by inappropriate response to EBV infection, usually leading to B cell lymphoma. The absence of iNKT cells reveals a role for SAP and XIAP in the regulation of iNKT cell development and implies the contribution of this cell subset to the control of infections and cancer progression. In keeping with the iNKT cell immunoregulatory role, their absence has been recently described in the Omenn syndrome, a primary immunodeficiency characterized by severe autoimmune manifestations (7). All these evidences have prompted us to investigate iNKT cells in Wiskott-Aldrich syndrome (WAS), a primary immunodeficiency associated with thrombocytopenia, recurrent infections, increased risk of developing cancer (mainly B cell lymphoma EBV associated), and autoimmunity (8, 9). WAS is caused by mutations in the gene encoding for the WAS protein (WASp), a key regulator of actin-dependent processes in hematopoietic cells (9). In humans, complete lack of WASp gives rise to the severe WAS phenotype, whereas hypomorphic mutations allowing residual WASp expression usually lead to X-linked thrombocytopenia (XLT), a milder disease characterized by marginal immune defects (8).

Thus far, many cellular defects resulting from the absence of WASp have been described, revealing the involvement of this protein in regulation of migration, cell trafficking and immunological synapse (IS) formation in distinct immune cell types (9). Aside from its role in actin cytoskeleton remodeling, WASp is required in signaling pathways downstream from NK and T cell activation (10–12). Although impaired innate and adaptive immune cell function can account for infections and partially explain the increased susceptibility to developing cancer and autoimmunity, a full comprehension of the cellular mechanisms underlying the pathogenesis of this syndrome still needs to be achieved (9, 13).

In the present work, we provide evidence that iNKT cells are absent in full-blown WAS patients. Moreover, analysis of iNKT cells in *was*$^{-/-}$ mice revealed defects in iNKT cell maturation and function. Our data point to a new role for WASp as an important regulator of iNKT cell development and function and lead to the hypothesis that these defects could contribute to the immune dysregulation in WAS.

RESULTS AND DISCUSSION

WAS patients, but not XLT patients, lack circulating iNKT cells

To investigate whether WASp deficiency has an impact on the amount of circulating human iNKT cells, we examined peripheral blood from three patients with a mild clinical phenotype (score of 1–2, XLT), six patients with severe WAS (score 3–5), and 13 age-matched healthy donors (HDs; Table S1). By flow cytometry, iNKT cells were nearly undetectable in WAS patients with a severe score (Fig. 1 A). Interestingly, three patients with XLT presented a detectable number of iNKT cells, even though their median value was within the lower range of HDs (Fig. 1 B). Therefore, impairment in iNKT cell number correlates with the severity of disease. In severe WAS patients lacking iNKT cells, the frequency of T cells expressing either TCR Vα24 or Vβ11, although variable, was in the range of HDs, suggesting that these TCR V regions can be used by WASp-deficient T cells (Fig. S1). Moreover, the analysis of the TCR Vβ repertoire in three WAS patients (WAS1, WAS28, and WAS33) and one case of XLT (WAS30) revealed the presence of all TCR Vβ families, with some

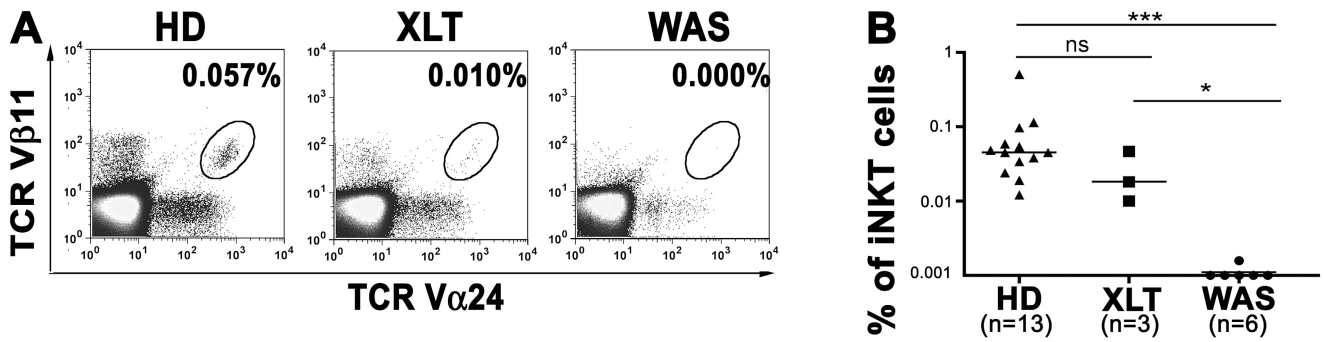

Figure 1. Lack of iNKT cells in WAS patients. (A) Representative flow cytometric analysis of peripheral blood iNKT cells from an age-matched HD control (HD), an XLT patient (XLT), and a WAS patient (WAS). Cells in the density plots are gated on CD3$^+$ cells. Percentages of iNKT cells (TCR-Vβ11$^+$ TCR-Vα24$^+$) are indicated. (B) Frequency of iNKT cells from 13 controls, 3 XLT, and 6 WAS patients. Bars represent the median value of each group. ns, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$.

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alterations probably caused by infections, which are frequent in these patients (Table S2). These findings indicate a profound defect in iNKT cell population, despite minor alterations in the TCR repertoire of mainstream T cells.

iNKT cell absence could contribute to the increased susceptibility of patients to pathogen infections. Indeed, lack of iNKT cells can impair the immune protection against viral, bacterial, and fungal infections (3). Furthermore, iNKT cells play a key role in tumor immunosurveillance, as suggested by the increased susceptibility of NKT-deficient mice to developing cancer (4) and a decreased iNKT cell number in patients with advanced cancer (14, 15). Therefore, we hypothesize that the absence of iNKT cells might contribute to the high susceptibility to develop EBV$^+$ B cell lymphoma observed in WAS patients, as has also been suggested for XLP (6).

Impaired iNKT cell development in *was*$^{-/-}$ mice

Given the paucity of material derived from WAS patients, we further investigated the role of WASp in the development and function of iNKT cells in *was*$^{-/-}$ mice (16). This mouse model recapitulates important immune system alterations of the human WAS (16). We first investigated the number and distribution of iNKT cells in the thymus, liver, and spleen by flow cytometric analysis. The percentage of iNKT cells stained specifically with α-GalCer–loaded CD1d tetramers was not significantly decreased in *was*$^{-/-}$ thymi, whereas it was two- and threefold reduced in spleen and liver, respectively, of *was*$^{-/-}$ animals compared with WT controls (Fig. 2 A and not depicted). However, the absolute number of iNKT cells was significantly reduced in all three compartments of *was*$^{-/-}$ mice in comparison with WT mice (Fig. 2 B).

Based on CD4 expression, two iNKT cell subsets can be distinguished: CD4$^+$ and CD4$^-$ CD8$^-$ double-negative (DN) cells (1). We analyzed whether the lack of WASp could affect the development and/or tissue distribution of one particular iNKT cell subset. Comparison of the absolute number of iNKT cells in *was*$^{-/-}$ and WT animals revealed a significant reduction of both CD4$^+$ and DN iNKT cells in *was*$^{-/-}$ mice in all tissues, with the exception of DN cells in the spleen. However no skewing toward one particular subset was observed (Fig. 2 C).

These findings indicate that the lack of WASp causes a significant decrease of iNKT cell number. We thus investigated whether the reduction of iNKT cells in *was*$^{-/-}$ mice might be the result of an altered thymic development. We first analyzed the expression of CD1d, which is required for iNKT cell positive selection (17), on DP lymphocytes without finding any alteration in *was*$^{-/-}$ mice in comparison with WT mice (Fig. S2).

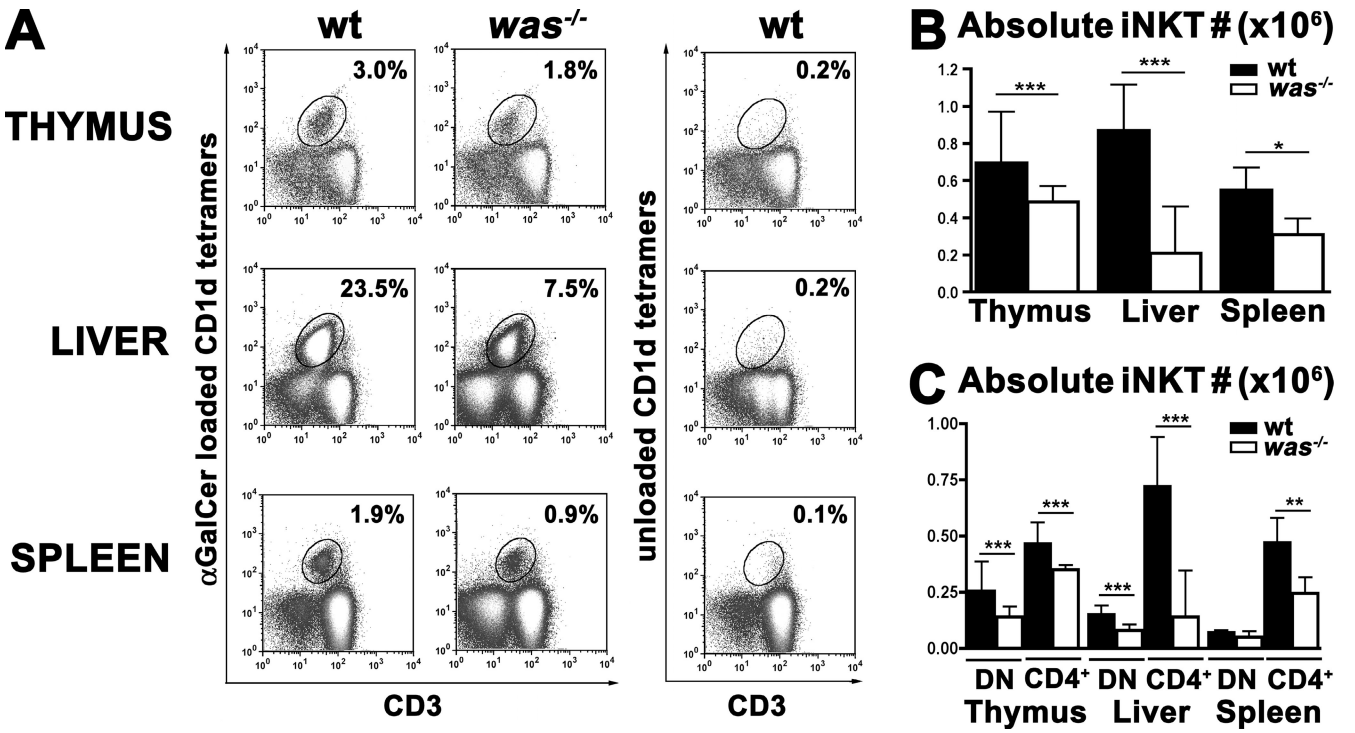

Figure 2. Reduced iNKT cell number in *was*$^{-/-}$ mice. (A) iNKT cells were analyzed by flow cytometry in thymus, liver, and spleen of C57BL/6 WT and *was*$^{-/-}$ mice (*was*$^{-/-}$). Thymocytes were stained with anti-CD8, anti-CD3 mAbs, and CD1d tetramers (α-GalCer loaded or unloaded), whereas hepatic leukocytes and splenocytes were stained with anti-B220 and anti-CD3 mAbs and CD1d tetramers (α-GalCer loaded or unloaded). After gating on CD8$^-$ or B220$^-$ cells, iNKT cells were identified as CD3$^+$ CD1d tetramers$^+$ cells. (B) Absolute numbers of iNKT cells were determined by multiplying their percentage by the absolute cell count within each sample. In A and B, data are representative of at least 10 mice per group analyzed in three independent experiments. (C) iNKT cells were stained with anti-CD4 antibodies. Comparison of the absolute number of CD4$^+$ or CD4$^-$ (DN) iNKT cells in thymus, liver, and spleen in WT versus *was*$^{-/-}$ mice is shown. In B and C, error bars represent the median and interquartile ranges of eight mice per group. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

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We next investigated the maturation steps of iNKT cells after positive selection, using CD44 and NK1.1 markers. As shown in Fig. 3 A, *was*$^{-/-}$ iNKT cells were mainly arrested at CD44^{+} NK1.1^{-} stage, and only a minor fraction became NK1.1^{+} both in the thymus and in periphery. The analysis of the absolute number of iNKT cells during the different maturation steps confirmed that WASp does not affect the earliest developmental phase, when *was*$^{-/-}$ iNKT cells are normally present (Fig. 3 B). Conversely *was*$^{-/-}$ iNKT cells accumulate at stage 2 (CD44^{+} NK1.1^{-}) without progressing to stage 3 (CD44^{+}NK1.1^{+}), suggesting a potential role of WASp in regulating the late phases of the differentiation process.

Among the events contributing to the final maturation of iNKT cells in the periphery, a crucial role seems to be played by CD1d recognition because NK1.1^{-} iNKT cells fail to properly complete their maturation in the absence of CD1d (18). To address this point, we examined CD1d expression in the periphery of *was*$^{-/-}$ mice, finding expression levels comparable to those of the WT (Fig. S2). These data rule out the possibility that an altered CD1d expression in the periphery may have a role in developmental block of *was*$^{-/-}$ iNKT cells.

Next, to assess whether the developmental defect of iNKT cells in *was*$^{-/-}$ mice is cell autonomous, we generated mixed BM chimeras by reconstituting irradiated CD45.1 mice with a mixture of equal numbers of CD45.2 lineage-negative (lin^{-}) cells from the BM of *was*$^{-/-}$ and WT mice. This way, CD45.2 *was*$^{-/-}$ iNKT cell precursors would develop in the presence of donor-derived CD45.2 DP thymocytes and hematopoietic cells from either *was*$^{-/-}$ or WT mice. In case of an iNKT cell-intrinsic defect, the presence of WT cells would not be sufficient to rescue the development of *was*$^{-/-}$ iNKT cells. BM chimeras were analyzed 7–9 wk after transplantation and evaluated for the expansion and maturation of iNKT cells derived from either *was*$^{-/-}$ or WT hematopoietic precursors. As shown in Fig. 4 A, the percentage of iNKT cells developing in *was*$^{-/-}$ mice reconstituted with *was*$^{-/-}$ lin^{-} cells (WAS$^{-/-}$→WAS$^{-/-}$ control) was reduced both in the thymus and, more markedly, in the periphery when compared with that observed in WT recipients transplanted with WT precursors (WT→WT control). Both mixed BM chimeras generated in *was*$^{-/-}$ and WT recipients displayed a level of iNKT cells similar to the WT→WT control, suggesting the same capacity of WT and was$^{-/-}$ recipients to support iNKT cell generation. The analysis of maturation of donor iNKT cells based on NK1.1 expression confirmed the complete developmental block of *was*$^{-/-}$ iNKT cells in WAS$^{-/-}$→WAS$^{-/-}$ controls as opposed to successful maturation of WASp^{+} iNKT cells in the WT→WT control (Fig. 4 B). In the

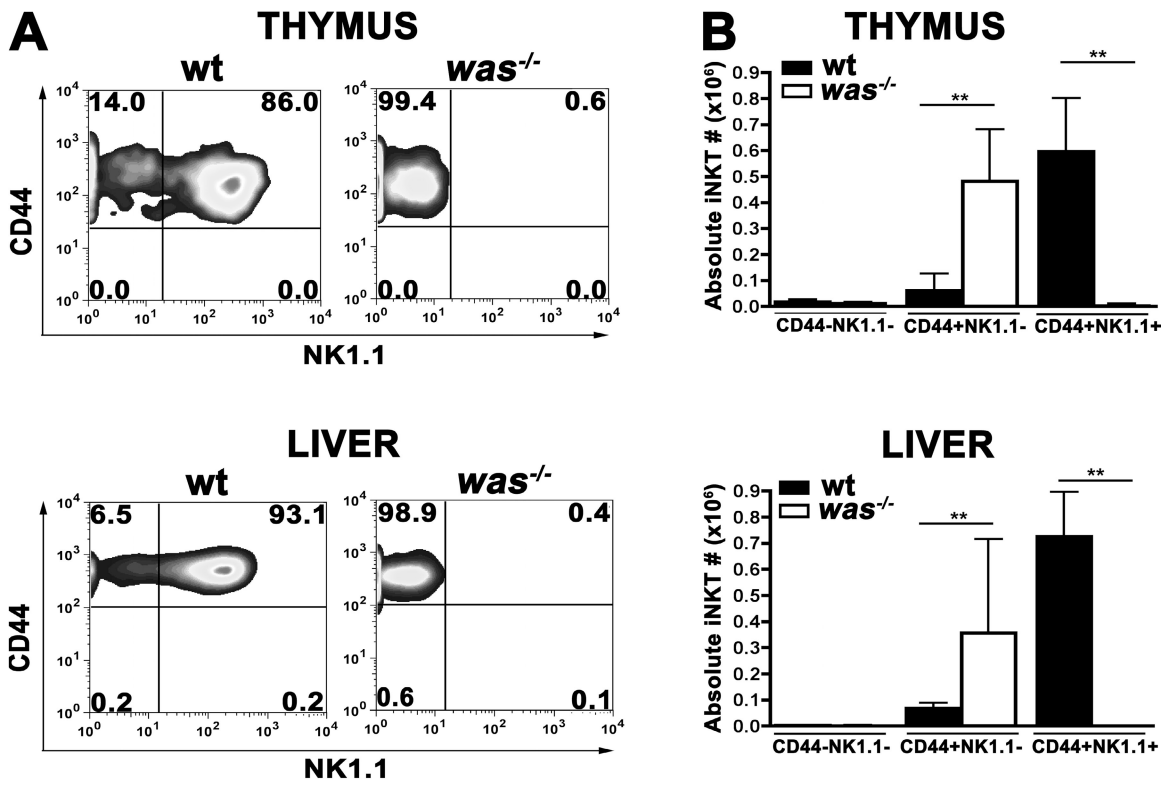

Figure 3. Block iNKT cell maturation in the absence of WASp. (A) Thymocytes and hepatic leukocytes from WT and *was*$^{-/-}$ mice were stained with anti-CD8, anti-CD3, anti-CD44, and anti-NK1.1 mAbs and α-GalCer–loaded CD1d tetramers. Maturation of iNKT cells (CD1d tetramer^{+}, CD3^{+}, and CD8^{-}) was assessed by CD44 and NK1.1 expression. Data are representative of six mice per group analyzed in two independent experiments. (B) Absolute numbers of iNKT cells (CD1d tetramer^{+}, CD3^{+}, and CD8^{-}) in thymus and liver of WT and *was*$^{-/-}$ mice. Error bars represent median and interquartile range of six mice per group. **, $P < 0.005$.

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BM chimeras, although WASp$^+$ iNKT cells could acquire the mature phenotype in both recipients, WASp$^-$ iNKT cells were unable to up-regulate NK1.1, even in the presence of hematopoietic cells derived from WT progenitors. Altogether, these findings demonstrate that the lack of WASp determines an iNKT cell–autonomous defect that impairs the maturation, survival, and/or expansion of these cells.

Many players regulating iNKT cell terminal maturation are molecules involved in the TCR signaling. For instance, a lower iNKT generation and maturation was observed in the absence of PKC-θ, a signal transduction molecule which is known to play an important role in the TCR–NF-κB pathway (19). It is of note that PKC-θ–mediated phosphorylation can activate WASp at the IS (20). Thus, in iNKT cell development, WASp

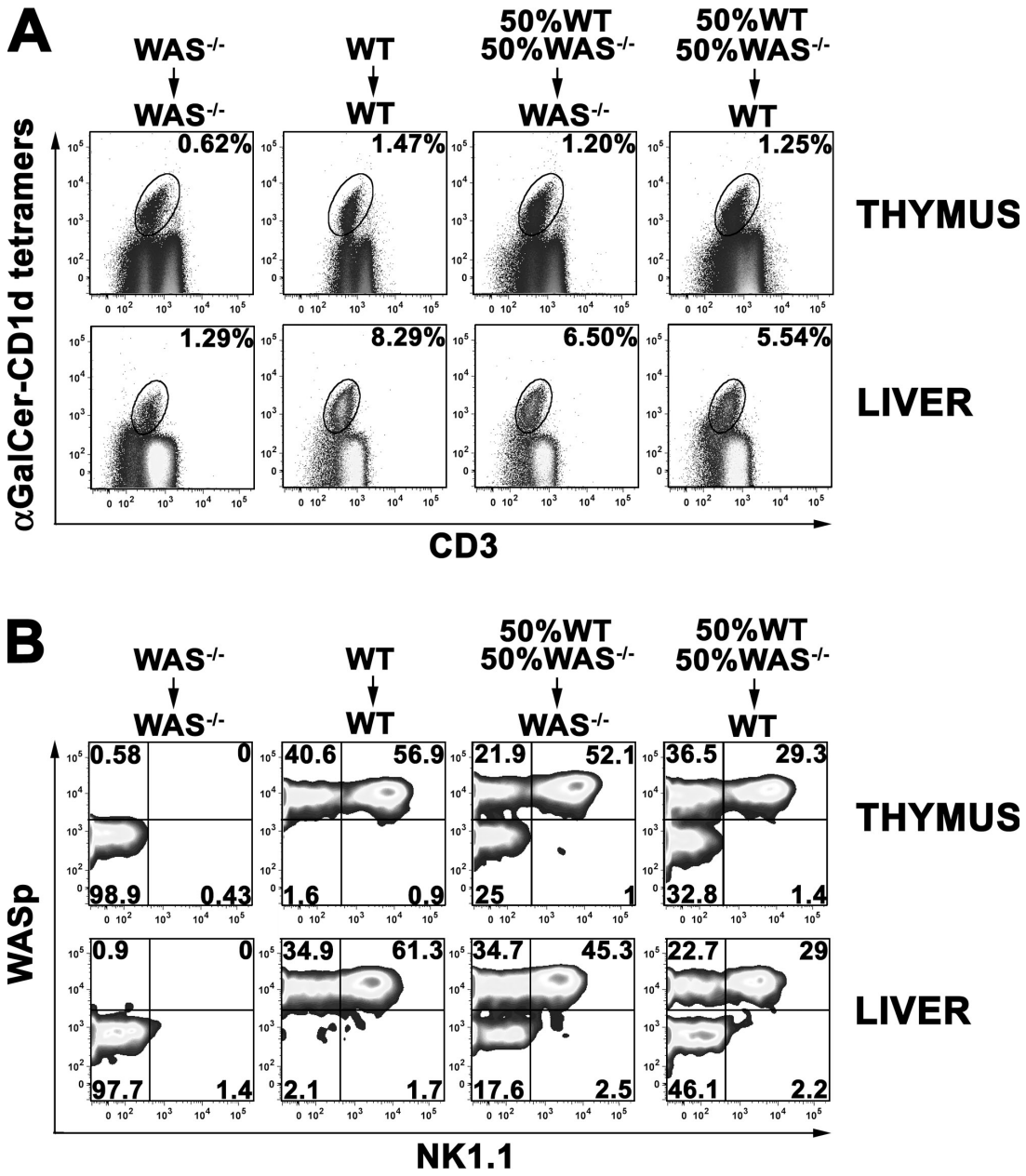

Figure 4. Cell-autonomous developmental defect of *was*$^{-/-}$ iNKT cells. (A) iNKT cells were analyzed by flow cytometry in thymus and liver of WT or *was*$^{-/-}$ recipient mice (CD45.1) transplanted with *was*$^{-/-}$ lin$^-$ cells, WT lin$^-$ cells, or a mixture of 50% WT and 50% *was*$^{-/-}$ lin$^-$ obtained from CD45.2 mice. Thymocytes and hepatic leukocytes were surface stained with α-GalCer–loaded CD1d tetramers, with anti-CD3 and anti-CD8 (thymocytes) or anti-B220 (hepatic leukocytes) mAbs. The percentage of iNKT cells (CD8$^-$ or B220$^-$ CD3$^+$CD1d tetramer$^+$ cells) is indicated in each plot. (B) Maturation of iNKT cells in thymus and liver of BM chimera mice. After gating on donor CD45.2$^+$, iNKT cells were further analyzed for NK1.1 and WASp expression. The percentage of mature (NK1.1$^+$) and immature (NK1.1$^-$) iNKT cells from WT donors (WASp$^+$) or from *was*$^{-/-}$ donors (WASp$^-$) is indicated in each plot. Data are representative of at least three mice per group from two independent experiments.

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might be a relevant target of the activated PKC-θ in the TCR–NF-κB pathway. In addition, iNKT cell development requires TCR-induced transcriptional factors, such as NF-κB, AP-1, and T-bet (2), whose expression or function are altered in the absence of WASp (10–12). Indeed, a reduced iNKT cell generation and maturation was associated with the absence of the transcriptional factor NF-κB (21), and WASp was demonstrated, at least in NK cells, to be involved in the regulation of NF-κB nuclear translocation (10). Moreover, transgenic mice overexpressing BATF, a negative regulator of AP-1 activity, showed defective maturation of iNKT cells (22, 23). Interestingly, WASp absence was associated with a lower AP-1 binding activity in mouse T cells (12). A severe block in NK1.1 expression was also reported in mice lacking T-bet, a transcriptional factor associated to Th1 immunity (24). A possible role for WASp in the regulation of T-bet is supported by a recent study from our group showing a reduced T-bet expression in TCR-stimulated $CD4^+$ T cell lines from WAS patients (11). Furthermore, very recent evidences have highlighted the critical role of costimulatory signals arising from B7–CD28 interaction in promoting the expansion of mature $NK1.1^+$ iNKT cells (25, 26). Interestingly, WASp was demonstrated to be required for a normal ligation-induced CD28 endocytosis, a process which is relevant to CD28 costimulatory functions (27). These evidences support the hypothesis that WASp acts as an important component in the downstream events of TCR pathway during iNKT cell terminal maturation.

Impaired function of *was*$^{-/-}$ iNKT cells

To investigate whether iNKT cells also exhibit functional defects in the absence of WASp, we evaluated the in vivo response to α-GalCer, a synthetic glycosphingolipid that elicits a prompt and selective activation of iNKT cells through the CD1d molecule. Upon α-GalCer stimulation, iNKT cells quickly produce huge amounts of Th1 and Th2 cytokines. We injected 1 μg α-GalCer i.v. into *was*$^{-/-}$ mice and WT controls and evaluated IL-4 and IFN-γ production in the sera of treated animals 3, 6, 12, and 24 h after stimulation. Although in *was*$^{-/-}$ mice the kinetics of cytokine production was similar to that in WT mice, the amount of IL-4 and IFN-γ in *was*$^{-/-}$ sera was significantly reduced at every time point that was tested (Fig. 5 A). The lower cytokine levels could be a result of the reduced iNKT cell number in *was*$^{-/-}$ mice but also of the impaired ability of *was*$^{-/-}$ iNKT cells to produce cytokines. To address this issue, we administered α-GalCer to *was*$^{-/-}$ or WT mice and analyzed ex vivo the intracellular cytokine production by iNKT cells. 45 min after α-GalCer injection, a significant fraction of WT iNKT cells produced a considerable amount of intracellular IL-4 and IFN-γ,

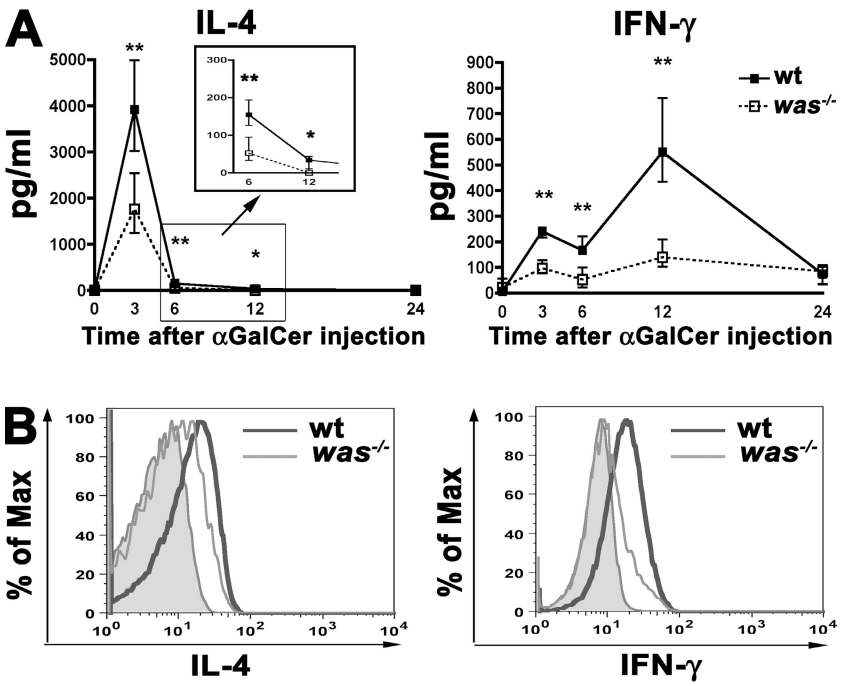

Figure 5. Impaired cytokine production by *was*$^{-/-}$ iNKT cells. (A) In vivo IL-4 and IFN-γ production upon α-GalCer administration in WT and *was*$^{-/-}$ mice. Sera were analyzed at 3, 6, 12, and 24 h upon injection. The graphs show the amount of cytokines produced by six WT and six *was*$^{-/-}$ mice. Black and white squares represent the median values of WT and *was*$^{-/-}$ mice groups, respectively. The vertical bars represent the interquartile range of each group. *, $P < 0.05$; **, $P < 0.005$. (B) IL-4 and IFN-γ production in WT and *was*$^{-/-}$ mice at the single iNKT cell level. WT and *was*$^{-/-}$ mice were injected with α-GalCer and, after 45 min, hepatic leukocytes were isolated and stained with α-GalCer–loaded CD1d tetramers and anti-CD3, anti–IL-4, and anti–IFN-γ mAbs. Representative analysis of IL-4 and IFN-γ intracellular production by iNKT cells ($CD3^+$ and CD1d $tetramer^+$) from WT (thick line) and *was*$^{-/-}$ (thin line) mice is shown. Filled histograms represent IL-4 or IFN-γ production by untreated WT mice. Data are from one representative experiment of three.

whereas *was*$^{-/-}$ iNKT cells showed an impaired production of both cytokines (Fig. 5 B). In parallel, we evaluated the in vivo iNKT cell expansion induced by α-GalCer injection. The analysis of iNKT cell number at different time points (3, 7, and 11 d) revealed, as expected, a peak of expansion of WT iNKT cell at day 3 followed by a contraction at day 7. Conversely, *was*$^{-/-}$ iNKT cells showed a delayed kinetics with maximum expansion at day 7 followed by a contraction at day 11 (Fig. S3 A). The increase of *was*$^{-/-}$ iNKT cell absolute number induced by α-GalCer was significant compared with the untreated mice. However, the capacity of *was*$^{-/-}$ iNKT cells to expand was substantially lower than WT iNKT cells, as shown by the differences in fold increase values (Fig. S3 B).

The functional defect of *was*$^{-/-}$ iNKT cells was further confirmed by their inability to help antigen-specific B cell responses in vivo compared with WT-activated iNKT cells (28). The functional impairment of *was*$^{-/-}$ iNKT cells upon in vivo activation may not be caused by their arrest at the immature stage 2 of differentiation because peripheral WT iNKT with immature phenotype are able to produce both Th1 and Th2 cytokines (29). On the contrary, it may result from various factors such as alterations in antigen presentation, an improper interaction between iNKT cells and APC, or a cell-autonomous defect.

Indeed, the activation of iNKT cells requires TCR recognition of a glycolipid antigens–CD1d complex on the surface of APCs (1). It is possible that a reduced antigen presentation ability of *was*$^{-/-}$ APC may contribute to the in vivo impaired functionality of *was*$^{-/-}$ iNKT cells because *was*$^{-/-}$ DC have a reduced ability to migrate, assemble podosomes, and process particulate antigens (9). Moreover, it is tempting to speculate that iNKT cells, like conventional T lymphocytes, require IS formation to achieve a proper activation. In the absence of WASp, this process has been demonstrated to be defective (30). Furthermore, altered activation of transcriptional factors NF-AT and AP-1, occurring in the absence of WASp (11, 12), may affect not only the development, as discussed in previous paragraphs, but also iNKT cell function. In particular, reduction in NF-AT activation and alteration in nuclear translocation correlates with the impaired IL-2 production observed in *was*$^{-/-}$ T lymphocytes (11, 12). In agreement with this hypothesis, mouse models carrying defects in molecules involved in the activation of AP-1 present alteration in iNKT cells including a perturbed cytokine profile (22).

Altogether, these results show that the lack of WASp leads to profound alterations in iNKT cells, which are absent in severe WAS patients and reduced in the *was*$^{-/-}$ mouse model. In *was*$^{-/-}$ mice, accumulation of immature iNKT cells (CD44^{+} NK1.1^{-}), together with the lack of mature subset, suggests a key role for WASp in iNKT cell maturation process. Moreover, analysis of in vivo stimulation and cytokine production reveals that peripheral iNKT cells are functionally impaired in *was*$^{-/-}$ mice. WASp is known as a central player in T cell activation by controlling actin polymerization, which in turn favors the generation of a long-lived IS between T cells and APCs (30, 31). We hypothesize that the postselection expansion and differentiation of iNKT cells requires the formation of a proper IS between developing iNKT cells and CD1d-expressing cells. The stable IS may allow developing iNKT cells to receive and integrate various agonist signals, originating from invariant TCR engagement and costimulatory molecules, to achieve full differentiation. Indeed, the defective *was*$^{-/-}$ iNKT cells resemble the ones generated in the absence of signal transduction molecules or transcription factors, such as PKC-θ, NF-κB, or T-bet. These molecules belong to the complex cascade of events generated upon TCR triggering and IS formation in conventional T cells and regulate iNKT cell expansion or survival, maturation, and cytokine production (19, 21, 24). Furthermore, the genetic deletion of costimulatory molecules, such as CD28, affects expansion and phenotypic and functional differentiation of iNKT cells (25, 26).

In conclusion, these findings provide the first evidence that WASp acts as an important player for the generation of mature and functional iNKT cells. Moreover, our data add a new perspective in the comprehension of the complex immune dysregulation and tumor susceptibility characterizing WAS.

MATERIALS AND METHODS

Patients. Blood samples from patients and age-matched HDs were obtained according to standard ethical procedures and with the approval of the San Raffaele Scientific Institute Internal Review Board (TIGET02).

Mice. C57BL/6 (B6) *was*$^{-/-}$ mice were provided by K.A. Siminovitch (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada) (16). CD45.1 *was*$^{-/-}$ mice were generated in our facility. B6 WT mice were purchased from Charles River Laboratories. All mice were 8–12 wk old. Experiments were performed according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute (IACUC318).

Cell preparation and flow cytometry. Human PBMCs were purified on Ficoll gradient (PoC; Axis-Shield) and stained with anti-CD4 (RPAT4; BD), anti-CD3 (UCHT1; BD), anti–TCR-Vα24 (C15; Beckman Coulter), and anti–TCR-Vβ11 (C21; Beckman Coulter) antibodies. For mouse studies, single cell suspensions were obtained from liver, spleen, and thymus. Hepatic leukocytes were purified using a Percoll (Sigma-Aldrich) gradient. The following mAbs were used for surface staining: anti-B220 (RA3-6B2), anti-CD8a (53–6.7), anti-CD4 (RM4-5), anti-CD44 (IM7), and anti-CD45.2 (104; all purchased from BD); anti-CD3 (17A2) and anti-NK1.1 (PK136; both purchased from BioLegend); and CD1d tetramers (ProImmune). For lipid loading, CD1d tetramers were incubated overnight with a 12 molar excess of α-GalCer (Axxora). Intracytoplasmic staining was performed using the Cytofix/Cytoperm kit (BD) and the following mAbs: anti–IFN-γ (XMG1.2) and anti–IL-4 (11B11; BD). Anti-WASp antibody was provided by H. Ochs (Research Center for Immunity and Immunotherapies, Seattle Children's Research Institute, University of Washington, Seattle, WA). Cells were acquired on a FACS CANTO (BD) and analyzed with FlowJo Software (Tree Star, Inc.).

Generation of BM chimeras. BM lin^{-} cells from CD45.2 B6 WT or *was*$^{-/-}$ mice were purified with the mouse hematopoietic progenitor enrichment kit (StemCell Technologies Inc.). *Was*$^{-/-}$ recipient mice (CD45.1) were irradiated (900 rad) before receiving i.v. 2.5×10^5 lin^{-} cells. Reconstitution was monitored by flow cytometry on blood cells. Mice were sacrificed 7–9 wk after transplantation for the analysis of iNKT cells in thymus and liver. Thymocytes were depleted of CD8^{+} cells by magnetic beads (Miltenyi Biotec) to enrich iNKT cell fraction.

In vivo activation and cytokine production. WT and *was*$^{-/-}$ mice were i.v injected with 1 μg α-GalCer in PBS or not injected as controls. To measure the in vivo IL-4 and IFN-γ production, blood samples were collected 3, 6, 12, and 24 h after injection. Serum cytokine levels were measured by Bio-Plex Technology (Bio-Rad Laboratories). To test the ex vivo

IL-4 and IFN-γ intracellular production by liver iNKT cells, mice were sacrificed 45 min after α-GalCer injection.

Statistical analysis. All data were analyzed with a two-tailed Mann-Whitney U test.

Online supplemental material. Table S1 describes the gene mutations and clinical status of patients. Table S2 shows the TCR-Vβ repertoire of HD and WAS patients. Fig. S1 displays the analysis of TCR-Vα24 and TCR-Vβ11 single-positive cells in HD, XLT, and WAS patients. Fig. S2 depicts CD1d expression in DP thymocytes and in splenic B cells and DCs. Fig. S3 shows iNKT cell in vivo expansion upon α-GalCer injection. Fig. S4 describes iNKT cell help to antigen-specific antibody responses. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081773/DC1.

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REFERENCES

1. Bendelac, A., P.B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25:297–336.
2. Godfrey, D.I., and S.P. Berzins. 2007. Control points in NKT-cell development. *Nat. Rev. Immunol.* 7:505–518.
3. Tupin, E., Y. Kinjo, and M. Kronenberg. 2007. The unique role of natural killer T cells in the response to microorganisms. *Nat. Rev. Microbiol.* 5:405–417.
4. Crowe, N.Y., M.J. Smyth, and D.I. Godfrey. 2002. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* 196:119–127.
5. Van Kaer, L. 2007. NKT cells: T lymphocytes with innate effector functions. *Curr. Opin. Immunol.* 19:354–364.
6. Latour, S. 2007. Natural killer T cells and X-linked lymphoproliferative syndrome. *Curr. Opin. Allergy Clin. Immunol.* 7:510–514.
7. Matangkasombut, P., M. Pichavant, D.E. Saez, S. Giliani, E. Mazzolari, A. Finocchi, A. Villa, C. Sobacchi, P. Cortes, D.T. Umetsu, and L.D. Notarangelo. 2008. Lack of iNKT cells in patients with combined immune deficiency due to hypomorphic RAG mutations. *Blood.* 111:271–274.
8. Imai, K., T. Morio, Y. Zhu, Y. Jin, S. Itoh, M. Kajiwara, J. Yata, S. Mizutani, H.D. Ochs, and S. Nonoyama. 2004. Clinical course of patients with WASP gene mutations. *Blood.* 103:456–464.
9. Ochs, H.D., and A.J. Thrasher. 2006. The Wiskott-Aldrich syndrome. *J. Allergy Clin. Immunol.* 117:725–738.
10. Huang, W., H.D. Ochs, B. Dupont, and Y.M. Vyas. 2005. The Wiskott-Aldrich syndrome protein regulates nuclear translocation of NFAT2 and NF-kappa B (RelA) independently of its role in filamentous actin polymerization and actin cytoskeletal rearrangement. *J. Immunol.* 174:2602–2611.
11. Trifari, S., G. Sitia, A. Aiuti, S. Scaramuzza, F. Marangoni, L.G. Guidotti, S. Martino, P. Saracco, L.D. Notarangelo, M.G. Roncarolo, and L. Dupre. 2006. Defective Th1 cytokine gene transcription in CD4+ and CD8+ T cells from Wiskott-Aldrich syndrome patients. *J. Immunol.* 177:7451–7461.
12. Cianferoni, A., M. Massaad, S. Feske, M.A. de la Fuente, L. Gallego, N. Ramesh, and R.S. Geha. 2005. Defective nuclear translocation of nuclear factor of activated T cells and extracellular signal-regulated kinase underlies deficient IL-2 gene expression in Wiskott-Aldrich syndrome. *J. Allergy Clin. Immunol.* 116:1364–1371.
13. Torgerson, T.R., and H.D. Ochs. 2007. Regulatory T cells in primary immunodeficiency diseases. *Curr. Opin. Allergy Clin. Immunol.* 7:515–521.
14. Tahir, S.M., O. Cheng, A. Shaulov, Y. Koezuka, G.J. Bubley, S.B. Wilson, S.P. Balk, and M.A. Exley. 2001. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J. Immunol.* 167:4046–4050.
15. Fujii, S., K. Shimizu, V. Klimek, M.D. Geller, S.D. Nimer, and M.V. Dhodapkar. 2003. Severe and selective deficiency of interferon-gamma-producing invariant natural killer T cells in patients with myelodysplastic syndromes. *Br. J. Haematol.* 122:617–622.
16. Zhang, J., A. Shehabeldin, L.A. da Cruz, J. Butler, A.K. Somani, M. McGavin, I. Kozieradzki, A.O. dos Santos, A. Nagy, S. Grinstein, et al. 1999. Antigen receptor–induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein–deficient lymphocytes. *J. Exp. Med.* 190:1329–1342.
17. Bendelac, A. 1995. Positive selection of mouse NK1$^+$ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med.* 182:2091–2096.
18. McNab, F.W., S.P. Berzins, D.G. Pellicci, K. Kyparissoudis, K. Field, M.J. Smyth, and D.I. Godfrey. 2005. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J. Immunol.* 175:3762–3768.
19. Stanic, A.K., J.S. Bezbradica, J.J. Park, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase C theta and NF-kappa B. *J. Immunol.* 172:4667–4671.
20. Sasahara, Y., R. Rachid, M.J. Byrne, M.A. de la Fuente, R.T. Abraham, N. Ramesh, and R.S. Geha. 2002. Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol. Cell.* 10:1269–1281.
21. Stanic, A.K., J.S. Bezbradica, J.J. Park, N. Matsuki, A.L. Mora, L. Van Kaer, M.R. Boothby, and S. Joyce. 2004. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. *J. Immunol.* 172:2265–2273.
22. Williams, K.L., A.J. Zullo, M.H. Kaplan, R.R. Brutkiewicz, C.D. Deppmann, C. Vinson, and E.J. Taparowsky. 2003. BATF transgenic mice reveal a role for activator protein-1 in NKT cell development. *J. Immunol.* 170:2417–2426.
23. Zullo, A.J., K. Benlagha, A. Bendelac, and E.J. Taparowsky. 2007. Sensitivity of NK1.1-negative NKT cells to transgenic BATF defines a role for activator protein-1 in the expansion and maturation of immature NKT cells in the thymus. *J. Immunol.* 178:58–66.
24. Townsend, M.J., A.S. Weinmann, J.L. Matsuda, R. Salomon, P.J. Farnham, C.A. Biron, L. Gapin, and L.H. Glimcher. 2004. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity.* 20:477–494.
25. Williams, J.A., J.M. Lumsden, X. Yu, L. Feigenbaum, J. Zhang, S.M. Steinberg, and R.J. Hodes. 2008. Regulation of thymic NKT cell development by the B7-CD28 costimulatory pathway. *J. Immunol.* 181:907–917.
26. Zheng, X., H. Zhang, L. Yin, C.R. Wang, Y. Liu, and P. Zheng. 2008. Modulation of NKT cell development by B7-CD28 interaction: an expanding horizon for costimulation. *PLoS One.* 3:e2703.
27. Badour, K., M.K. McGavin, J. Zhang, S. Freeman, C. Vieira, D. Filipp, M. Julius, G.B. Mills, and K.A. Siminovitch. 2007. Interaction of the Wiskott-Aldrich syndrome protein with sorting nexin 9 is required for CD28 endocytosis and cosignaling in T cells. *Proc. Natl. Acad. Sci. USA.* 104:1593–1598.
28. Galli, G., P. Pittoni, E. Tonti, C. Malzone, Y. Uematsu, M. Tortoli, D. Maione, G. Volpini, O. Finco, S. Nuti, et al. 2007. Invariant NKT cells sustain specific B cell responses and memory. *Proc. Natl. Acad. Sci. USA.* 104:3984–3989.
29. McNab, F.W., D.G. Pellicci, K. Field, G. Besra, M.J. Smyth, D.I. Godfrey, and S.P. Berzins. 2007. Peripheral NK1.1 NKT cells are mature and functionally distinct from their thymic counterparts. *J. Immunol.* 179:6630–6637.
30. Dupre, L., A. Aiuti, S. Trifari, S. Martino, P. Saracco, C. Bordignon, and M.G. Roncarolo. 2002. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity.* 17:157–166.
31. Sims, T.N., T.J. Soos, H.S. Xenias, B. Dubin-Thaler, J.M. Hofman, J.C. Waite, T.O. Cameron, V.K. Thomas, R. Varma, C.H. Wiggins, et al. 2007. Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. *Cell.* 129:773–785.

Evidence for Long-term Efficacy and Safety of Gene Therapy for Wiskott–Aldrich Syndrome in Preclinical Models

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Wiskott–Aldrich Syndrome (WAS) is a life-threatening X-linked disease characterized by immunodeficiency, thrombocytopenia, autoimmunity, and malignancies. Gene therapy could represent a therapeutic option for patients lacking a suitable bone marrow (BM) donor. In this study, we analyzed the long-term outcome of WAS gene therapy mediated by a clinically compatible lentiviral vector (LV) in a large cohort of *was*^{null} mice. We demonstrated stable and full donor engraftment and Wiskott–Aldrich Syndrome protein (WASP) expression in various hematopoietic lineages, up to 12 months after gene therapy. Importantly, we observed a selective advantage for T and B lymphocytes expressing transgenic WASP. T-cell receptor (TCR)-driven T-cell activation, as well as B-cell's ability to migrate in response to CXCL13, was fully restored. Safety was evaluated throughout the long-term follow-up of primary and secondary recipients of WAS gene therapy. WAS gene therapy did not affect the lifespan of treated animals. Both hematopoietic and non-hematopoietic tumors arose, but we excluded the association with gene therapy in all cases. Demonstration of long-term efficacy and safety of WAS gene therapy mediated by a clinically applicable LV is a key step toward the implementation of a gene therapy clinical trial for WAS.

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INTRODUCTION

Wiskott–Aldrich Syndrome (WAS) is a monogenic X-linked immunodeficiency also characterized by microthrombocytopenia, eczema, and a high susceptibility to develop tumors and multiple autoimmune manifestations.[1,2] WAS is caused by mutations in the *WAS* gene,[3] which impairs or abolishes the expression of the Wiskott–Aldrich Syndrome protein (WASP). WASP is expressed in hematopoietic cells,[4] where it integrates several extracellular stimuli to control actin cytoskeleton reorganization[5] and signal transduction.[6] WASP deficiency impairs several immune cell functions such as *in vivo* leukocyte migration,[7] pathogen killing by natural killer cells[8] and neutrophils,[9] antigen presentation by antigen-presenting cells,[10] homing of B cells to secondary lymphoid organs leading to dysfunctional humoral responses,[11] immunological synapse formation and T-cell activation after T-cell receptor (TCR)-engagement.[6,12–15] Thus, the wide range of hematopoietic cell types affected by the absence of WASP indicates that a valid therapeutic approach should target hematopoietic stem cells (HSCs).

Currently, the only resolutive therapeutic option for WAS patients is bone marrow transplantation (BMT) from related human leukocyte antigen-identical or matched unrelated donor.[16–18] Because it is crucial to proceed with BMT before the progressive worsening of the clinical status,[19] patients lacking a related identical donor or a matched unrelated donor often undergo BMT from a mismatched related donor. However, mismatched related donor transplantation is associated with an elevated risk of developing life-threatening Epstein–Barr virus lymphoproliferative syndrome, infections, autoimmunity, graft rejection, and graft-versus-host disease[16] resulting in reduced survival.[16–18] In the past few years, retroviral vector–mediated gene therapy emerged as a valid therapeutic alternative for patients with primary immunodeficiencies that could not benefit from conventional therapies.[20,21] Unfortunately, the occurrence of hematopoietic malignancies or preleukemic clonal expansions due to insertional mutagenesis was observed in clinical gene therapy trials for X-linked severe combined immunodeficiency and X-linked chronic granulomatous disease, respectively,[22] indicating the need of safer tools for gene transfer. Compared to the retroviral vectors used in the first clinical studies, lentiviral vectors (LVs) transduce nondividing HSCs

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effectively,[23] lack transcriptionally active long terminal repeats, and are less genotoxic.[24] We previously developed a human *WAS* promoter/cDNA encoding LV (w1.6W) that was successfully used to restore WASP expression in CD34$^+$ HSCs, T cells, B cells, and dendritic cells, and to correct TCR-driven activation in T-cell lines derived from WAS patients.[13,25] Moreover, the *in vivo* efficacy of this vector was demonstrated in a gene therapy setting in nonlethally irradiated *was*^{null} mice, with evidence of multilineage WASP expression in hematopoietic cells and correction of TCR-driven activation of splenic T cells.[26] However, gene therapy treated mice were followed up for 4 months, a period that is insufficient to investigate the long-term stability of WASP expression and T-cell functional correction, and the safety of the gene therapy approach. Indeed, it has been shown that severe adverse events related to gene therapy in mice may develop after a follow-up >7 months,[27,28] or after secondary BMTs.[29]

In this study, we analyzed the stability of WASP expression and the persistence of T- and B-cell functional correction in a large cohort (n = 68) of *was*^{null} mice treated with w1.6W-mediated gene therapy and followed up for 12 months. Safety was assessed by long-term monitoring of tumor incidence in primary (n = 68) and secondary (n = 32) recipients of WAS gene therapy, using two distinct models of *was*^{null} mice. We found that the w1.6W vector promoted long-term WASP expression in many hematopoietic cell lineages, which was associated to the selective advantage of WASP$^+$ T and B lymphocytes. In addition, WAS gene therapy corrected T-cell activation defects, normalized B-cell migration in response to CXCL13, and did not cause any severe adverse event. These results are instrumental for the implementation of an LV-mediated gene therapy clinical trial in WAS patients.

RESULTS

Transplantation of LV-transduced lin$^-$ *was*^{null} cells

To investigate the long-term efficacy and safety of WAS gene therapy in mice, we purified lineage marker–depleted (lin$^-$) cells from bone marrow (BM) BL6-*was*^{null} mice, and transduced them *ex vivo* by a single hit of a human *WAS* promoter/cDNA containing LV (w1.6W), at either low (10–20) or high (200) multiplicity of infection (MOI). Untransduced *was*^{null} and wt lin$^-$ cells were used as control (lin$^-$ *was*^{null} and lin$^-$ wt groups, respectively). The w1.6W LV integrated into *was*^{null} lin$^-$ cells and promoted the expression of transgenic WASP in a dose-dependent fashion (**Figure 1a**). Transduced or control lin$^-$ cells were injected in sex- or CD45 allele-mismatched BL6-*was*^{null} mice conditioned by sublethal irradiation. Donor cell engraftment was analyzed 1 year after transplantation by Y-chromosome specific real-time PCR (**Figure 1b**), or by flow cytometric detection of the CD45.2 allele (**Figure 1c**). High and stable engraftment (ranging 69–100%) was achieved in all hematopoietic cell types isolated from mice belonging to all experimental groups. These results demonstrate that sublethal irradiation of the recipient *was*^{null} mice is sufficient to ensure robust and persistent donor cell engraftment.

Analysis of long-term WASP expression in multiple hematopoietic cell lineages

To assess whether differentiated hematopoietic cells from gene therapy treated *was*^{null} mice expressed WASP 12 months after

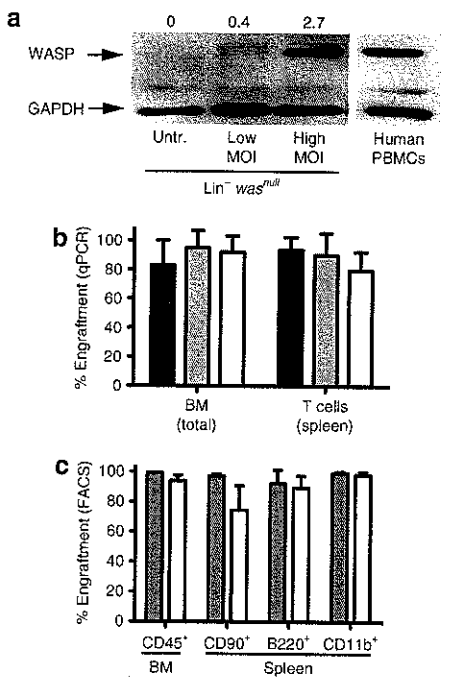

Figure 1 Transplantation of w1.6W-transduced lin$^-$ *was*^{null} cells for long-term experiments. (**a**) Western blot analysis of WASP expression in lysates of lin$^-$ *was*^{null} cells either untransduced or transduced with the w1.6W vector at low or high MOI. As control, WASP expression in human PBMCs is depicted. Numbers indicate VCN. (**b**) Donor cell engraftment measured by real-time PCR in the indicated cell types, 12 months after transplantation of transduced or control lin$^-$ cells. Mean ± SD is depicted. Lin$^-$ wt n = 8, black bars; low MOI n = 11, light grey bars; lin$^-$ *was*^{null} n = 4, empty bars. (**c**) Donor cell engraftment measured by flow cytometry analysis, 12 months after transplantation of transduced or control lin$^-$ cells. Mean ± SD is depicted. High MOI n = 17, dark grey bars; lin$^-$ *was*^{null} n = 4, empty bars. MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; VCN, vector copy number; WASP, Wiskott–Aldrich Syndrome protein.

treatment, we performed flow cytometric analysis. We detected WASP expression in BM CD45$^+$ cells, and in splenic myeloid cells (CD11b$^+$), B cells (B220$^+$), CD8$^+$ T cells, and CD4$^+$ T cells (**Figure 2a**), thus demonstrating effective long-term multilineage activity of the w1.6W vector *in vivo*. In all cell types analyzed, the proportion of WASP-expressing cells was higher in the high MOI group, as compared to the low MOI group (**Figure 2b,c**). Interestingly, in both MOI groups, the proportion of WASP-expressing splenic B cells, CD8$^+$ T cells, and CD4$^+$ T cells was significantly higher than that of BM CD45$^+$ cells and splenic myeloid cells (**Figure 2b,c**). Consistently, vector copy number (VCN) in splenic donor T cells was significantly higher than VCN in total donor BM cells, in both the low MOI (2.0 ± 0.8 versus 1.0 ± 0.4, $P < 0.05$) and the high MOI gene therapy group (4.2 ± 0.9 versus 2.7 ± 0.7, $P < 0.05$). Taken together, these data demonstrate the long-term expression of transgenic WASP in multiple cell lineages and strongly suggest the occurrence of a selective advantage for WASP-expressing lymphocytes.

Complete blood cell counts 12 months after gene therapy

Twelve-months-old BL6-*was*^{null} mice display B lymphopenia, thrombocytopenia, reduced monocyte counts, and modest

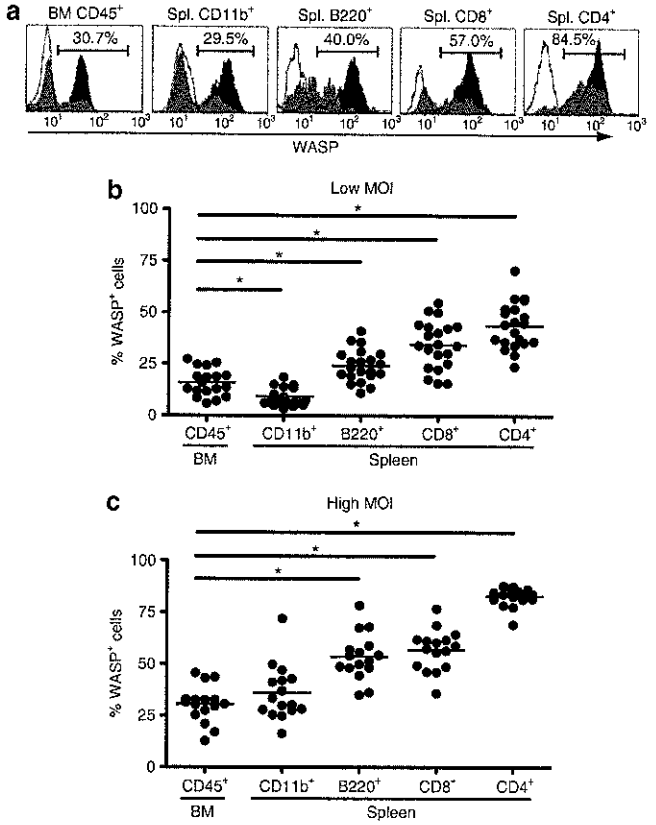

Figure 2 WASP expression in hematopoietic cells 12 months after gene therapy. (**a**) Analysis of WASP expression in BM CD45$^+$ cells, and splenic CD11b$^+$, B220$^+$, CD8$^+$, and CD4$^+$ cells. Histograms report WASP expression in a representative *was*^{null} (white), wt (black) and high MOI gene therapy treated mouse (gray). Numbers represent the percentage of WASP$^+$ cells in the high MOI gene therapy treated mouse. (**b**,**c**) Cumulative analysis of the percentage of WASP$^+$ cells in the indicated cell types within the low MOI gene therapy group (**b**, $n = 21$) or the high MOI gene therapy group (**c**, $n = 16$). Dots represent values from each single mouse. Bars indicate the mean value of the distribution. *$P < 0.05$, Student *t*-test. MOI, multiplicity of infection; WASP, Wiskott–Aldrich Syndrome protein.

granulocytosis (**Table 1**). To assess whether WAS gene therapy could restore normal hematopoietic cell counts, we performed complete blood cell counts on gene therapy treated animals and controls. Despite very high donor engraftment (**Figure 1b**), leukocyte, erythrocyte, and platelet counts in the lin$^-$ wt group were lower than those in nonirradiated wt mice (**Table 1**). Therefore, to evaluate the efficacy of WAS gene therapy, we took as reference the groups transplanted with wt or *was*^{null} lin$^-$ cells. The absolute count of T cells, B cells, and platelets in the lin$^-$ *was*^{null} group was decreased as compared to the lin$^-$ wt group, although granulocyte count was increased (**Table 1**). Mice treated with high vector MOI had B cell, platelet, and granulocyte counts comparable to those of the lin$^-$ wt group and significantly different from those of lin$^-$ *was*^{null} group. Conversely, mice belonging to the low vector MOI group had B cell, platelet, and granulocyte counts significantly different from those of the lin$^-$ wt group and similar to those of lin$^-$ *was*^{null} group (**Table 1**). T-cell counts in the lin$^-$ wt group were increased above levels of nonirradiated wt mice, although in the other groups they were comparable to levels found in nonirradiated *was*^{null} mice. Taken together, these data suggest that high MOI gene therapy could be more efficacious than low MOI gene therapy in ameliorating B cell, platelet, and granulocyte counts.

Long-term correction of T-cell activation after WAS gene therapy

The main consequence of WASP deficiency in T cells is impaired proliferation and cytokine production in response to TCR triggering.[6,13–15,30,31] After *in vitro* TCR stimulation, the proliferative response and the secretion of interleukin (IL)-2, interferon-γ, tumor necrosis factor-α, IL-4, and IL-10 were strongly reduced in *was*^{null} T cells, as compared to wt T cells (**Figure 3a–f**). WAS gene therapy performed at both high and low MOI completely corrected these defects, while transplantation of untransduced lin$^-$ *was*^{null} cells was ineffective (**Figure 3a–f**). The degree of functional correction was independent of the MOI used for initial transduction of lin$^-$ *was*^{null} cells. This indicates that WASP expression in 34–44% of splenic T cells (as found in the low MOI group, see **Figure 2b**) is sufficient to correct *was*^{null} T-cell dysfunctions. These data are in agreement with those observed in short-term gene therapy studies in *was*^{null} mice,[26,31–33] and further extend the efficacy of WAS gene therapy to a much longer time frame. Moreover, we provide the first demonstration that WAS gene therapy can restore Th2 cytokine secretion by T cells stimulated through the TCR.

Long-term correction of B-cell migration after WAS gene therapy

B lymphocytes isolated from *was*^{null} mice display impaired chemotaxis *in vitro*. This defect may contribute to decreased homing to B-cell areas of the spleen and to defective humoral immune responses *in vivo*.[11] To investigate the long-term functional correction of B cells after gene therapy, we measured migration of purified splenic B cells in response to the chemokine CXCL13 using a transwell system. Basal B-cell migration was comparable in all experimental groups (**Figure 4**). When exposed to CXCL13, the percentage of migrating wt B cells was 34.0 ± 5.2%, while it was only 23.0 ± 5.1% for *was*^{null} B cells (**Figure 4**). After WAS gene therapy performed at low MOI, migration of B cells in response to CXCL13 was normalized (**Figure 4**). This finding provides evidence of long-term efficacy of WAS gene therapy in restoring B-cell migratory potential.

Twelve-month follow-up of gene therapy treated mice

To evaluate safety of the WAS gene therapy approach, we periodically checked the general health status of WAS gene therapy treated BL6-*was*^{null} mice and controls. Survival curves of animals belonging to the high and low MOI groups were statistically similar to those of control lin$^-$ *was*^{null} and lin$^-$ wt groups, thus excluding a detrimental impact of lentiviral transduction and *WAS* transgene expression on overall survival (**Figure 5a**).

Evaluation of tumor occurrence could be performed in 32/34 mice in the lin$^-$ *was*^{null} group (94%), in 46/48 mice belonging to the low MOI gene therapy group (96%), in 18/20 mice in the high MOI gene therapy group (90%), and in 15/15 mice in the lin$^-$ wt group (100%). Overall tumor incidence 12 months after treatment was comparable in the gene therapy and control groups (**Figure 5b**). The occurrence of nonhematopoietic tumors (gut adenocarcinoma,

Table 1 Blood cell counts 12 months after gene therapy

Group	*n*	WBC (×10⁶/ml)[a]	T cells (×10⁶/ml)[a]	B cells (×10⁶/ml)[a]	Monocytes (×10⁶/ml)[a]	Granulocytes (×10⁶/ml)[a]	RBC (×10⁹/ml)[a]	PLT (×10⁶/ml)[a]
Wt	13	11.3 ± 2.3	2.0 ± 0.7	5.6 ± 1.8	1.0 ± 0.2	1.7 ± 0.9	10.0 ± 0.8	1,304 ± 214
was^{null}	11	6.6 ± 2.0[b]	1.5 ± 0.5	1.2 ± 0.7[b]	0.5 ± 0.2[b]	2.8 ± 1.4[b]	9.8 ± 1.1	655 ± 154[b]
Lin⁻ wt	13	7.4 ± 2.9[b]	2.9 ± 1.1[b,c]	1.8 ± 0.9[b,c]	0.5 ± 0.2[b]	1.6 ± 0.8[c]	9.1 ± 0.7[b]	941 ± 189[b,c]
High MOI	17	5.3 ± 2.4[b,d]	1.2 ± 0.6[b,d,e]	1.8 ± 0.7[b,c,e]	0.6 ± 0.4[b]	1.1 ± 1.0[c,e]	8.3 ± 1.8[b]	803 ± 220[b,c,e]
Low MOI	38	5.7 ± 1.9[b]	1.7 ± 0.6[d]	1.0 ± 0.5[b,d]	0.5 ± 0.2[b]	2.3 ± 1.3[d]	9.3 ± 1.1[b]	551 ± 134[b,d]
Lin⁻*was*^{null}	20	6.8 ± 3.2[b]	1.4 ± 0.7[b,d]	1.0 ± 0.6[b,d]	0.7 ± 0.5	3.2 ± 2.5[b,d]	9.1 ± 0.8[b]	614 ± 214[b,d]

Abbreviations: MOI, multiplicity of infection; RBC, red blood cells; PLT, platelets; WBC, white blood cells.
[a]Average value ± SD. [b]$P < 0.05$ as compared to wt. [c]$P < 0.05$ as compared to lin⁻*was*^{null}. [d]$P < 0.05$ as compared to lin⁻ wt. [e]$P < 0.05$ as compared to low MOI.

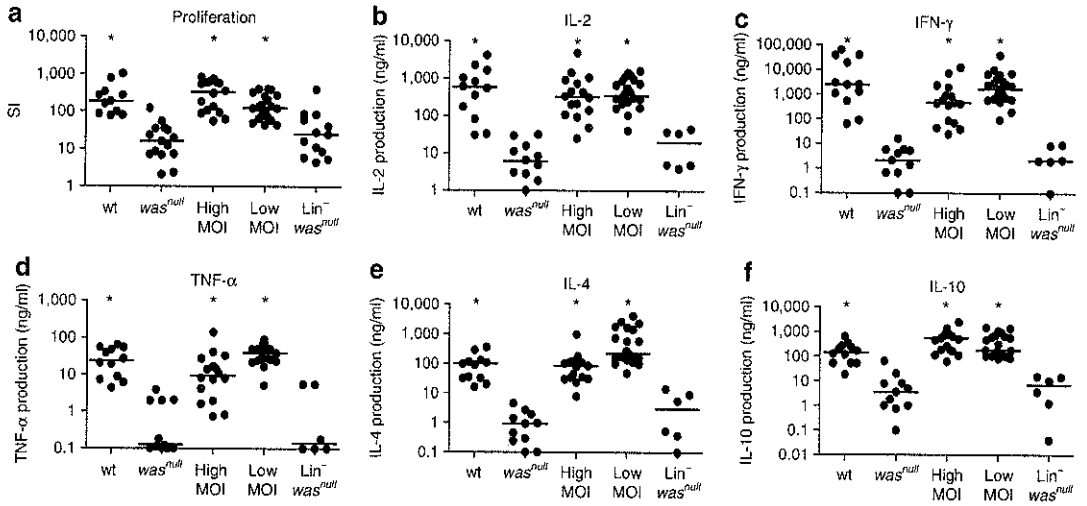

Figure 3 Long-term restoration of T-cell function after gene therapy. Splenic T cells were purified by immunomagnetic beads, and stimulated with 2 μg/ml anti-CD3 mAbs for 48 hours. (**a**) Proliferation was measured by ^{3}H-thymidine incorporation. wt $n = 11$; *was*^{null} $n = 15$; high MOI $n = 16$; low MOI $n = 23$; lin⁻ *was*^{null} $n = 13$. Results are expressed by the stimulation index (SI), *i.e.*, the ratio between cpm of stimulated and nonstimulated cells. (**b–e**) Cytokine levels were measured by BioPlex technology in conditioned supernatants. wt $n = 12$; *was*^{null} $n = 11$; high MOI $n = 16$; low MOI $n = 23$; lin⁻ *was*^{null} $n = 6$. Dots represent the measurement performed in each single mouse. Bars represent the median value. *$P < 0.05$ as compared to *was*^{null} group, Mann–Whitney test. cpm, counts per minute; MOI, multiplicity of infection.

hepatocarcinoma, ovary tumor, poorly differentiated thoracic carcinoma, eye carcinoma, skin carcinoma) was observed in four mice belonging to the lin⁻ *was*^{null} group (12.5%), three mice from the high MOI gene therapy group (16.7%), four mice from the low MOI gene therapy group (8.7%), and three mice (20.0%) belonging to the lin⁻ wt group (**Figure 5b** and **Supplementary Table S1**). As these tumors were of nonhematopoietic origin, it could be excluded that they were caused by gene therapy. Lymphomas were observed in two mice belonging to the lin⁻ *was*^{null} group (6.3%), and in four mice (8.7%) of the low MOI gene therapy group (**Figure 5b** and **Supplementary Table S1**). In all cases, real-time PCR analysis proved that they were of host origin and that they lacked LV integrations, thus excluding a role of gene therapy in the genesis of those hematopoietic malignancies (Table 2). In conclusion, our data generated in a large cohort of gene therapy treated mice followed up for 12 months strongly support the safety of the proposed gene therapy approach.

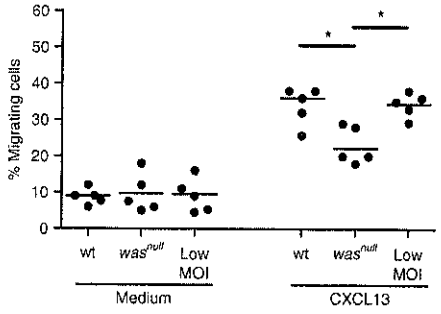

Figure 4 Long-term correction of B-cell migration after gene therapy. B cells were purified from the spleen of gene therapy treated or control mice. Migration through 5-μm pore transwells was measured in response to medium only or medium supplemented with 1 μg/ml CXCL13. Results are expressed as the percentage of input cells that migrated to the lower well. Each dot represents a single mouse. Bars depict the mean value. $n = 5$ per group. *$P < 0.05$ as compared to the *was*^{null} group, Student *t*-test.

Evaluation of safety of WAS gene therapy through serial BMT

To complete our safety evaluation, we performed secondary transplantations of w1.6W-transduced BM cells using a different model of WAS gene therapy based on the 129-*was*^{null} mouse strain and lethal irradiation.[32] Lin⁻ cells purified from 129-*was*^{null} mice were transduced with one or two hits of the w1.6W LV (WA 1X and WA 2X groups, respectively), or with one hit of pGFP control

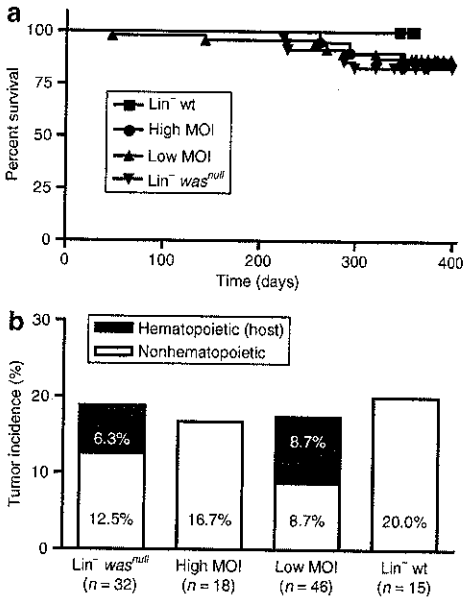

Figure 5 Twelve-month follow-up of gene therapy treated mice. (**a**) Survival curve of mice belonging to the lin⁻ wt (filled squares, n = 15), high MOI (filled circles, n = 20), low MOI (filled triangles, n = 48), and lin⁻ *was*^{null} (filled inverted triangles, n = 34) groups. The log-rank test failed to detect any statistically significant difference between the different groups. (**b**) Incidence of tumors in mice treated with gene therapy as compared to control groups. White bars represent the percentage of nonhematopoietic tumors, while black bars represent the percentage of host-derived hematopoietic tumors. Note that donor-derived hematopoietic tumors were never observed. Numbers represent the percentage of tumor incidence. MOI, multiplicity of infection.

vector (*was*^{null} GFP group). As further control, lin⁻ wt cells were transduced with one hit of pGFP vector (wt GFP group). Efficient w1.6W transduction of lin⁻ cells was demonstrated by high VCN and transgenic WASP expression (**Figure 6a**). Transduced lin⁻ cells were transplanted in lethally irradiated 129-*was*^{null} mice (11–12 per group, see **Supplementary Table S2**). Four months later, engraftment and median VCN of donor cells in the BM was comparable in all groups (**Figure 6b,c**). No tumor occurrence was observed in any recipient of primary graft (**Supplementary Table S2**).

To perform secondary transplantations, we selected 2–3 primary transplanted mice for each group, among those with the highest donor chimerism and/or VCN (**Figure 6b,c**). Two million total BM cells were transplanted in lethally irradiated, secondary 129-*was*^{null} recipients (5–6 for each donor mouse, 11–16 per group, see **Supplementary Table S3**). Secondary transplanted mice were followed up for 6 months. At killing, chimerism in the BM and spleen was variable but comparable to that in the BM of primary recipients (**Figure 6b**). VCN in the BM and spleen of secondary recipients was comparable to that in the BM of primary recipients in all groups except the WA 1X group, where a decreased VCN was observed in the BM only (**Figure 6c**). *WAS* mRNA expression was detected in the BM and spleen of mice belonging to the WA 1X and WA 2X groups (data not shown). Taken together, these results indicate that w1.6W-transduced HSCs engrafted in secondary recipients. Multilineage hematopoietic cell reconstitution in all groups was demonstrated by hemogram analysis (**Supplementary Table S3**)

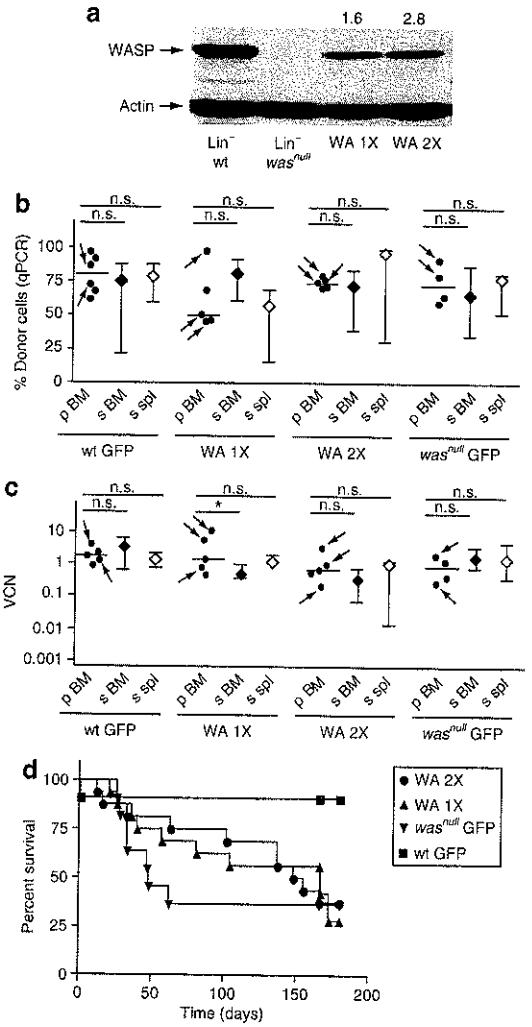

Figure 6 Follow-up of mice recipient of secondary bone marrow transplantation. (**a**) Western blot analysis of WASP expression in lysates of 129-*was*^{null} lin⁻ cells either untransduced or transduced with one (WA 1X) or two (WA 2X) hits of the w1.6W vector. As control, WASP expression in 129-wt lin⁻ cells is depicted. Numbers indicate VCN. (**b**) Engraftment of donor cells in sex-mismatched 129-*was*^{null} mice, as determined by Y chromosome-specific real-time PCR. For primary transplantations (p BM), dots represents measurements performed in total bone marrow cells, and horizontal bars depict the median value of the distribution. n = 4–6 mice per group. Arrows highlight mice chosen as donors for secondary transplants. For secondary transplantations, diamonds represent median ± interquartile range in total bone marrow cells (s BM, black) and spleen (s spl, white). wt GFP group: n = 10; WA 1X group: n = 9; WA 2X group: n = 9; *was*^{null} GFP group: n = 4. n.s. = not significant, Mann–Whitney test. (**c**) VCN in hematopoietic cells of mice recipient of either primary or secondary transplantation. VCN was determined by real-time PCR and normalized for the percentage of engraftment. For primary transplantations (p BM), dots represent measurements performed in total bone marrow cells, and horizontal bars depict the median value of the distribution. n = 4–5 mice per group. Arrows highlight mice chosen as donors for secondary transplants. For secondary transplantations, diamonds represent median ± interquartile range in total bone marrow cells (s BM, black) and spleen (s spl, white). wt GFP group: n = 10; WA 1X group: n = 9; WA 2X group: n = 8; *was*^{null} GFP group: n = 4. *P < 0.05, n.s. = not significant, Mann–Whitney test. (**d**) Survival curves of mice receiving secondary bone marrow transplantation. For wt GFP (filled squares), n = 11; for WA 1X (filled triangles), n = 16; for WA 2X (filled circles), n = 16; for *was*^{null} GFP (filled inverted triangle), n = 11. P < 0.05 for wt GFP group as compared to all the other groups, log-rank test. BM, bone marrow; VCN, vector copy number.

Table 2 Characteristics of hematopoietic tumors

Experiment	Mouse ID	Group	Histo-pathology	% Donor[a]	VCN[b]
12-month follow-up	645.6	Low MOI	Thymic lymphoma	2.5	0.04
12-month follow-up	648.4	Low MOI	Thymic lymphoma	2.3	0.1
12-month follow-up	663.1	Low MOI	Mesenteric LN lymphoma	1.1	0.1
12-month follow-up	664.3	Low MOI	Thymic lymphoma	0.7	0.03
12-month follow-up	611.2	lin$^-$ *was*^{null}	Thymic lymphoma	0.5	0.03
12-month follow-up	661.2	lin$^-$ *was*^{null}	Thymic lymphoma	1.8	ND
Secondary transplants	06-091-S20	WA 1X	Thymic lymphoblastic T-cell lymphoma	0.01	0.002
Secondary transplants	06-091-S25	WA 2X	Thymic early stage T-cell lymphoma	1	0.03
Secondary transplants	06-091-S24	WA 2X	Thymic lymphoblastic lymphoma	0	0

Abbreviations: LV, lentiviral vector; ND, not done; VCN, vector copy number.
[a]Percentage of donor cells as determined by Y-chromosome specific real-time PCR. [b]VCN per diploid genome as determined by LV specific real-time PCR. VCN was not normalized for the percentage of donor cells within the tumor.

and immunophenotyping of the BM and spleen (data not shown). Despite that the lifespan of secondary recipients of the *was*^{null} GFP, WA 1X, and WA 2X groups was lower than that of wt GFP group, while survival of mice belonging to the WA 1X and WA 2X groups was comparable to that of the *was*^{null} GFP group (**Figure 6d**). Evaluation of tumor occurrence could be performed in 7/11 mice in the *was*^{null} GFP group (64%), in 12/16 mice belonging to the WA 1X group (75%), in 13/16 mice in the WA 2X group (81%), and in 10/11 mice in the wt GFP group (91%). Based on macroscopic and histological examination, we observed no tumors in secondary recipients of wt GFP and *was*^{null} GFP cells (**Supplementary Table S3**). On the other hand, one secondary recipient belonging to the WA 1X group (8.3%) and two secondary recipients of the WA 2X group (15.4%) developed thymic lymphomas (**Table 2** and **Supplementary Table S3**). Molecular analysis indicated that all three lymphomas were of host origin and did not contain LV sequences (**Table 2**). In conclusion, serial transplantation experiments followed up for a cumulative period of 10 months did not reveal any toxicity related to the usage of the w1.6W LV.

DISCUSSION

In this study, we investigated the long-term efficacy and safety of WAS gene therapy using a *WAS* promoter/cDNA containing LV (w1.6W), compatible with future clinical application. *In vivo* WAS gene therapy studies performed up to now have addressed restoration of T-cell function in small cohorts of mice, with a maximal follow-up of 7 months.[26,31–33] By observing a large cohort (n = 68) of gene therapy treated *was*^{null} mice for 12 months, we could demonstrate the long-term stability of transgene expression, the occurrence of a selective advantage for WASP-expressing T and B lymphocytes, the persistence of T- and B-cell functional correction, and the improvement of B lymphocyte and platelet counts. Importantly, no donor-derived, LV-containing hematopoietic tumor developed in primary (n = 68) and secondary (n = 32) recipients of w1.6W-transduced BM cells, indicating that WAS gene therapy was very well tolerated.

An important rationale for the use of LVs is their high efficiency at transducing HSCs after a short *ex vivo* infection, which could favor the maintenance of stem cell properties.[23] Moreover, LVs are less genotoxic than LTR-active retroviral vectors,[24] and can harbor tissue specific internal promoters. Indeed, we and others have developed LVs expressing human WASP under the control of a 1.6 kb[34] or 0.5 kb[35] fragment of the proximal *WAS* promoter (w1.6W[13] and w0.5W[35] LVs, respectively). This strategy can allow the fine regulation of WASP expression in hematopoietic cells while avoiding toxicity due to off-target transgene expression.[13,36] Because WASP expression is regulated at both transcriptional and post-translational levels by highly specific interactions with transcription factors[25] and WIP,[37] respectively, it was crucial to compare WASP expression elicited by w0.5W and w1.6W LVs in human cells. Both w0.5W and w1.6W were comparable to various LVs containing constitutive promoters in eliciting WASP expression in T cells, B cells and CD34$^+$ HSCs derived from WAS patients, and in restoring the function of T cells and dendritic cells.[13,25] In addition, clonal analysis of LV-transduced, WAS patient-derived CD4$^+$ T-cell clones indicated that 1–2 copies of w1.6W and w0.5W LVs were sufficient to restore physiological WASP expression (**Supplementary Figure S1**). The w1.6W and the w0.5W LVs were also comparable in restoring T-cell activation and podosome formation in dendritic cells after gene therapy in *was*^{null} mice (**Supplementary Figures S2 and S3**). However, the w1.6W LV retained several transcription factor binding sites,[25] and was therefore selected for further preclinical development.

In accordance to our previous data,[26] the w1.6W LV-transduced murine lin$^-$ stem/progenitor cells at high efficiency after a short *ex vivo* infection. The stem cell properties of HSCs contained in the lin$^-$ population were likely well preserved, as suggested by durable engraftment of LV-transduced hematopoietic cells for at least 12 months in primary recipients, and for at least 6 months in secondary recipients (**Figures 1b,c** and **6b,c**). Importantly, a sublethal irradiation was enough to allow persistent multilineage engraftment of donor cells. This finding is in line with an anecdotal report about a WAS patient who successfully underwent matched

unrelated donor BMT after reduced intensity conditioning,[38] and suggests that reduced intensity conditioning could also be exploited as a less toxic preparatory regimen before administration of gene therapy to WAS patients.

One of the key issues for the success of gene therapy is whether WASP expression leads to a selective advantage of WASP$^+$ hematopoietic cells *in vivo*, causing the replacement of WASP-null cells in the case of mixed chimerism. In this work, we documented an *in vivo* enrichment for both T and B lymphocytes expressing WASP after gene therapy (**Figures 2b,c**). Our finding is in line with previous investigations demonstrating the enrichment of WASP$^+$ lymphocytes in *was* heterozygous female mice,[39] in *was*^{null} mice undergoing competitive BMT,[31] in WAS patients developing mixed chimerism after BMT,[40] in revertant WAS patients,[41] and in heterozygous female carriers.[42] The enrichment of WASP$^+$ lymphocytes could be due to the rescue of a developmental dysfunction in T and B cells,[39] and to a peripheral growth/survival advantage caused by preferential homing to secondary lymphoid organs,[7,11] efficient TCR-driven expansion of T cells,[12,13] and resistance to spontaneous apoptosis.[43] In spite of the strong enrichment of WASP-expressing lymphocytes, mixed chimerism could persist after gene therapy and could be a concern for the development of autoimmunity as recently reported in a cohort of WAS patients treated by allogeneic BMT.[19] However, the latter situation is complicated by allogeneic reactions between the host and donor cells, differently from gene therapy, which relies on autologous transplantation. In addition, we have evidences that gene therapy performed in 129-*was*^{null} mice prevents the onset of autoimmune colitis.[32,44]

Of note, low MOI WAS gene therapy was sufficient to correct T- and B-cell functional defects, suggesting that a clinical benefit could be obtained even in case of partial correction of WASP expression. This is in line with a retrospective study that correlated residual WASP expression in WAS patients with minimal immune dysfunction, lower clinical score, and extended lifespan.[45] Undoubtedly, highly efficient gene therapy, represented in this study by the high MOI group, broadens the number of corrected defects. Indeed, B cell and platelet counts in the high MOI group increased to levels reached by *was*^{null} mice transplanted with lin$^-$ wt cells (**Table 1**). Accordingly, high doses of WASP-encoding LVs were necessary to obtain functional correction of dendritic cells after WAS gene therapy,[32,46] while low LV doses were ineffective.[32] This defect could also be alleviated by high MOI gene therapy mediated by the w1.6W vector (**Supplementary Figure S3**). The average VCN in the high MOI gene therapy group was 2 in the BM and 4 in T cells, which might be considered high if it is directly translated to patients. However, the w1.6W was designed for optimal performance in human cells, where we expect to obtain physiological WASP levels with VCN 1–2 (refs. 13,25 and **Supplementary Figure S1**). Alternatively, sequences of the *WAS* distal promoter might be inserted to further improve the performance of the w1.6W LV, because this was recently reported to improve the performance of the shorter w0.5 promoter.[47]

Importantly, we found substantial evidence supporting the safety of WAS gene therapy mediated by the w1.6W LV. Indeed, 12-month survival of primary grafted mice of the low and high MOI gene therapy groups was comparable to that of mice belonging to lin$^-$ *was*^{null} and lin$^-$ wt control groups (**Figure 5a**). We observed a similar incidence of tumors (that were mainly of nonhematopoietic origin) in gene therapy treated mice as compared to controls (**Figure 5b**). Occurrence of host-derived hematopoietic tumors is a well-known confounding effect in the evaluation of preclinical models of gene therapy.[48] Therefore, molecular analysis is essential to determine the relationship between vector transduction and transgene expression with oncogenesis. In our study, lymphomas arose in four mice (8.7%) of the low MOI gene therapy group and in two mice (6.3%) of the lin$^-$ *was*^{null} group (**Figure 5b** and **Supplementary Table S1**), but were all of host origin and did not bear LV integrations, as confirmed by molecular analysis (**Table 2**). Mice were followed for 12 months upon transplantation, a time window shorter as compared to a clinical follow-up but long enough to observe adverse events in murine models of gene therapy for common-γ chain or CD40L deficiencies.[27,28] In contrast, in our study none (0/68) of the primary grafted animals developed donor-derived, LV-transduced hematopoietic tumors up to 12 months after gene therapy. In some experimental settings, oncogenicity of gene transfer could be evidenced by secondary BMT.[29] However, in our secondary transplantation experiments spanning a period of 10 months, transduction of HSCs with the w1.6W LV did not cause tumorigenesis. Indeed, survival of *was*^{null} mice that received secondary transplantation of w1.6W-trasduced BM cells was comparable to that of *was*^{null} mice receiving secondary transplantation of GFP-transduced BM cells (**Figure 6d**). Although we documented the occurrence of 3 lymphomas out of 25 evaluable secondary recipients of w1.6W-transduced BM (12%, see **Supplementary Table S3**), they were of host origin and did not bear LV integrations (**Table 2**). Noteworthy, in our study we evaluated a total amount of integration events, calculated as being about 63×10^6, comparable to a single patient's dose (estimated as 75×10^6 events for a patient of 15 kg receiving 5×10^6 CD34$^+$ cells/kg with an average VCN = 1).

In addition to the experimental data we provide in this work, other considerations account for the safety of a WAS gene therapy approach using LVs. Indeed, *WAS* is not classified as a cancer related gene (negative search in the Cancer Gene Expression Database, http://lifesciencedb.jp/cged/, and in the Retrovirus Tagged Cancer Gene Database, http://rtcgd.abcc.ncifcrf.gov/, accessed November 2008), and WASP expression levels are controlled by post-transcriptional mechanisms.[49] Moreover, LVs offer a better safety profile as compared to retroviral vectors.[24] Taken together, these data strongly account for the safety of WAS gene therapy mediated by the w1.6W LV. The demonstration of long-term efficacy and safety of WAS gene therapy using the w1.6W LV presented in this work is critically contributing to the implementation of a multicentre WAS gene therapy clinical trial.

MATERIALS AND METHODS

Mice. C57BL/6 *was*^{null} mice (BL6-*was*^{null})[15] were crossed with C57BL/6-CD45.1 mice to obtain the BL6-*was*^{null}-CD45.1 strain at the San Raffaele Scientific Institute animal facility in Milan, Italy. This strain is characterized by long lifespan after sublethal irradiation and by marked thrombocytopenia, and therefore it was chosen for long-term observation studies and for the evaluation of platelet counts after gene therapy. A distinct *was*^{null} mouse model under the 129sv background (129-*was*^{null}) was kindly provided by S.B. Snapper,[14] and housed in Généthon (Evry, France).

This strain has a shorter lifespan due to colitis exacerbation after irradiation, and was therefore used for serial grafts. Control wt mice were purchased from Charles River Laboratories (Calco, Italy) or Iffa Credo (L'Arbesle, France). All mice used in this study were housed in specific pathogen-free conditions and subjected to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute or by Généthon.

LVs. The w1.6W vector is a self-inactivating LV constructed on the pRRL backbone and encoding for WASP as the only transgene.[13,25,26] The transcription of human *WAS* cDNA is controlled by a 1.6 kb sequence of the autologous proximal *WAS* promoter.[34] A Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) sequence was included in the vector, aiming at increasing transgene expression by transcript stabilization. The w1.6W vector is VSVg pseudotyped. The final configuration of the w1.6W vector, that will be used for large scale good manufacturing procedures production oriented to clinical application, was obtained by swapping it on the pCCL backbone, and substituting the wild-type WPRE with a mutated WPRE sequence (WPREmut6) lacking the transcription of a fragment of the potentially oncogenic woodchuck hepatitis virus-X protein.[50] The performance of the w1.6W vector was unaltered by those changes (S. Charrier and A. Galy, unpublished results), and both were used in this study. A GFP-encoding LV (pGFP) was used as control in some experiments. Two additional pRRL vectors encoding WASP from the w0.5 promoter or from the PGK promoter were used in supplemental studies and have been described earlier.[25] For this study, nongood manufacturing procedures small scale LV productions were made by co-transfection of transgene-encoding transfer plasmids with packaging plasmids encoding *gag/pol*, *VSV-G env*, and *rev* into 293T cells. Harvested virus particles were concentrated by ultracentrifugation. Virus p24 concentration was measured by enzyme-linked immunosorbent assay. Viral titer was determined by infection of HCT116 cells with serially diluted virus preparations followed by proviral sequence detection, as previously described.[32]

Immunophenotype and western blot analysis. The following mAbs were used for surface staining: anti-CD3 (17A2), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b/Mac1 (M1/70), anti-CD45R/B220 (RA3-6B2), anti-CD45.2 (104), anti-CD90.2 (53-2.1), all from BD Pharmingen (San Diego, CA). Intracytoplasmic detection of human and murine WASP was performed using the anti-WASP antibody 503 (a kind gift of H. Ochs and L.D. Notarangelo) after fixation and permeabilization of the cells using the Cytofix/Cytoperm kit (BD Pharmingen). Western blot analysis was performed as previously described.[32] Membranes were probed with the B9 or H250 anti-WASP Abs (crossreactive between human and murine WASP; Santa Cruz Technologies, Santa Cruz, CA), the anti-β-actin mAbs AC-15 (Sigma, St Louis, MO), and the MAB374 anti-GAPDH mAbs (Chemicon, Temecula, CA).

Real-time PCR analysis. Genomic DNA was extracted from hematopoietic cells using the QIAmp DNA Blood mini-kit (Qiagen, Hilden, Germany). DNA extraction from paraffin-embedded samples was performed either using the QIAmp DNA FFPE Tissue Kit (Qiagen) or the Agilent protocol available online (http://www.chem.agilent.com/cag/prod/dn/G4410-90020_CGH_Protocol_FFPE1_0.pdf). LV copy number (VCN) per diploid genome, and percentage of cells carrying the Y chromosome, were assessed by absolute quantification using the primers listed in **Supplementary Table S4.** Measurement of the human WAS mRNA relative to that of the murine TF2D mRNA was performed as previously reported.[32]

Long-term follow-up experiments. Long-term follow-up experiments were performed using BL6-*was*^{null} and wt mice. Lineage marker–depleted BM (lin$^-$) cells were purified and transduced using a previously reported protocol.[26] Transduction was performed by culturing 1×10^6 lin$^-$ cells in the presence of $1–2 \times 10^7$ or 2×10^8 infectious viral genomes (ig) per ml of culture (MOI = 10–20 and 200, respectively) for 12 hours. Lin$^-$ cells ($0.25–1 \times 10^6$ cells/mouse) were transferred intravenously into sublethally irradiated (700 rad) 6- to 8-week-old *was*^{null} sex- or CD45 allele-mismatched recipient mice. Some transduced lin$^-$ cells were grown *in vitro* for more than 7 days to analyze transduction efficiency. Twelve months after transplantation, splenic T cells were isolated by means of immunomagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and stimulated for 48 hours with plate-bound anti-CD3 mAbs (clone 17A2 from BD Pharmingen, 2 μg/ml) as previously described.[26] Proliferation was measured by ^{3}H-thymidine pulsing and liquid scintillation counting. For each sample, the stimulation index (*i.e.*, the ratio between counts per minute of stimulated versus nonstimulated cells) was calculated. Concentration of IL-2, interferon-γ, tumor necrosis factor-α, IL-4, and IL-10 was assessed by BioPlex technique (Bio-Rad Laboratories, Hercules, CA) in conditioned supernatants. To assess B-cell migration in response to CXCL13 (Peprotech, Rocky Hill, NJ), CD45R/B220$^+$ splenic B cells were selected by means of immunomagnetic beads (Miltenyi) and seeded in duplicate on the upper well of a 5-μm transwell (Corning Costar, Corning, NY) at the concentration of $1–2 \times 10^6$/ml in 100 μl of medium. In the bottom well, 600 μl of medium supplemented with 1 μg/ml of CXCL13 were placed. Cells were incubated for 6 h at 37 °C, and cells migrated to the lower well were counted. Percentage of migration was calculated as compared to the input cell number.

Secondary transplantation experiments. Secondary transplantation experiments were performed using 129-*was*^{null} or wt mice. Lin$^-$ cells were purified and cultured as previously reported,[32] with minor modifications. After 1 day of cytokine prestimulation, male *was*^{null} lin$^-$ cells were infected with the w1.6W vector at the concentration of 1×10^8 ig/ml (MOI = 100) for 6 hours, and then washed. Part of the cells received a second vector hit (MOI = 100) administered overnight, and then washed. As control, male *was*^{null} or wt lin$^-$ cells were transduced once with the pGFP vector (1×10^8 ig/ml, MOI = 100). One day after infection, lin$^-$ cells (0.5×10^6 cells/mouse) were transferred intravenously into *was*^{null} sex-mismatched female mice, which had been lethally irradiated (950 rad) 2–3 hours prior to transplantation. Some of the transduced lin$^-$ cells were cultured *in vitro* for 7 days for analysis of transduction efficiency. Four months after primary graft, mice with the highest VCN or Y chimerism in peripheral blood were chosen as BM donors for secondary transplants. Total BM cells were collected and transplanted (2×10^6 cells/mouse) into lethally irradiated (950 rad) female secondary *was*^{null} recipients. Mice were followed up for 6 months.

Animal follow-up, hemogram analysis, and histopathologic evaluation. Animals were weekly evaluated to monitor their general health status. At the end of programmed follow-up, or in case of compromised health status, mice underwent complete blood cell count and were euthanized to perform full macroscopic and histopathologic analysis of hematopoietic and nonhematopoietic tissues. For hemogram analysis in the long-term follow-up experiments, blood samples were collected in 4.5% EDTA and analyzed with a KX21N counter (Sysmex, Kobe, Japan). Absolute numbers of T cells, B cells, monocytes, and granulocytes were determined as reported previously.[26] For secondary transplants, blood was collected in 3.8% citrate solution, and analyzed using an MS9.3 counter (Schloessing Melet, Cergy-Pontoise, France). For histopathologic evaluation, organs (thymus, colon, small intestine, spleen, lymph nodes, liver, kidney, and bone) of all killed mice were formalin-fixed and paraffin-embedded. Tissue sections (4 μm thick) were stained with hematoxylin and eosin and evaluated by certified pathologists with experience in rodent tissue analysis (M.P., F.S., C.D. in Milan and by Biodoxis, an independent pathologist subcontractor, in Evry). Tumor samples were subjected to qPCR analysis, to assess their donor or host origin and the presence of integrated LV sequences.

Statistical analysis. In case of Gaussian distribution of the data (checked by Kolmogorov–Smirnov test), experimental groups were compared with a two-tailed Student *t*-test. Otherwise, a two-tailed Mann–Whitney test was used. P values <0.05 were considered significant.

SUPPLEMENTARY MATERIAL

Table S1. Lifespan, details of necropsy and histopathologic analysis, and blood cell counts of all BL6-*was*^{null} mice followed up for 12 months after gene therapy.

Table S2. Lifespan, details of necropsy and histopathologic analysis, and blood cell counts of all primary grafted 129-*was*^{null} mice.

Table S3. Lifespan, details of necropsy and histopathologic analysis, and blood cell counts of all secondary grafted 129-*was*^{null} mice.

Table S4. The list of primers for real-time PCR used in this study.

Figure S1. The analysis of WASP expression and vector copy number in WAS patient's CD4$^+$ T cell clones, after transduction with three WAS-encoding vectors (in which WASP is controlled by the PGK, the w1.6 or the w0.5 promoter).

Figure S2. The comparison between three WAS-encoding vectors (in which WASP is controlled by the PGK, the w1.6 or the w0.5 promoter) in the restoration of T cell activation after gene therapy.

Figure S3. The comparison between three WAS-encoding vectors (in which WASP is controlled by the PGK, the w1.6 or the w0.5 promoter) in the restoration of podosome formation by bone-marrow derived DCs.

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REFERENCES

1. Notarangelo, LD, Notarangelo, LD and Ochs, HD (2005). WASP and the phenotypic range associated with deficiency. *Curr Opin Allergy Clin Immunol* **5**: 485–490.
2. Ochs, HD and Thrasher, AJ (2006). The Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* **117**: 725–738.
3. Derry, J, Ochs, H and Francke, U (1994). Isolation of a novel gene mutated in Wiskott-Aldrich Syndrome. *Cell* **78**: 635–644.
4. Stewart, DM, Treiber-Held, S, Kurman, CC, Facchetti, F, Notarangelo, LD and Nelson, DL (1996). Studies of the expression of the Wiskott-Aldrich syndrome protein. *J Clin Invest* **97**: 2627–2634.
5. Kim, A, Kakalis, L, Abdul-Manan, N, Liu, G and Rosen, M (2000). Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature* **404**: 151–158.
6. Trifari, S, Sitia, G, Aiuti, A, Scaramuzza, S, Marangoni, F, Guidotti, LG *et al.* (2006). Defective Th1 cytokine gene transcription in CD4+ and CD8+ T Cells from Wiskott-Aldrich Syndrome patients. *J Immunol* **177**: 7451–7461.
7. Snapper, SB, Meelu, P, Nguyen, D, Stockton, BM, Bozza, P, Alt, FW *et al.* (2005). WASP deficiency leads to global defects of directed leukocyte migration *in vitro* and *in vivo*. *J Leukoc Biol* **77**: 993–998.
8. Gismondi, A, Cifaldi, L, Mazza, C, Giliani, S, Parolini, S, Morrone, S *et al.* (2004). Impaired natural and CD16-mediated NK cell cytotoxicity in patients with WAS and XLT: ability of IL-2 to correct NK cell functional defect. *Blood* **104**: 436–443.
9. Zhang, H, Schaff, UY, Green, CE, Chen, H, Sarantos, MR, Hu, Y *et al.* (2006). Impaired integrin-dependent function in Wiskott-Aldrich syndrome protein-deficient murine and human neutrophils. *Immunity* **25**: 285–295.
10. Bouma, G, Burns, S and Thrasher, AJ (2007). Impaired T-cell priming *in vivo* resulting from dysfunction of WASp-deficient dendritic cells. *Blood* **110**: 4278–4284.
11. Westerberg, L, Larsson, M, Hardy, SJ, Fernandez, C, Thrasher, AJ and Severinson, E (2005). Wiskott-Aldrich syndrome protein deficiency leads to reduced B-cell adhesion, migration, and homing, and a delayed humoral immune response. *Blood* **105**: 1144–1152.
12. Dupre, L, Aiuti, A, Trifari, S, Martino, S, Saracco, P, Bordignon, C *et al.* (2002). Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. *Immunity* **17**: 157–166.
13. Dupre, L, Trifari, S, Follenzi, A, Marangoni, F, Lain de Lera, T, Bernad, A *et al.* (2004). Lentiviral vector-mediated gene transfer in T cells from Wiskott-Aldrich syndrome patients leads to functional correction. *Mol Ther* **10**: 903–915.
14. Snapper, S, Rosen, F, Mizoguchi, E, Cohen, P, Khan, W, Liu, C-H *et al.* (1998). Wiskott-Aldrich Syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* **9**: 81–91.
15. Zhang, J, Shehabeldin, A, da Cruz, LA, Butler, J, Somani, AK, McGavin, M *et al.* (1999). Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J Exp Med* **190**: 1329–1342.
16. Filipovich, A, Stone, J, Tomany, S, Ireland, M, Kollman, C, Pelz, C *et al.* (2001). Impact of donor type on outcome of bone marrow transplantation for Wiskott-Aldrich syndrome: collaborative study of the International Bone Marrow Transplant Registry and the National Marrow Donor Program. *Blood* **97**: 1598–1603.
17. Kobayashi, R, Ariga, T, Nonoyama, S, Kanegane, H, Tsuchiya, S, Morio, T *et al.* (2006). Outcome in patients with Wiskott-Aldrich syndrome following stem cell transplantation: an analysis of 57 patients in Japan. *Br J Haematol* **135**: 362–366.
18. Pai, SY, DeMartiis, D, Forino, C, Cavagnini, S, Lanfranchi, A, Giliani, S *et al.* (2006). Stem cell transplantation for the Wiskott-Aldrich syndrome: a single-center experience confirms efficacy of matched unrelated donor transplantation. *Bone Marrow Transplant* **38**: 671–679.
19. Ozsahin, H, Cavazzana-Calvo, M, Notarangelo, LD, Schulz, A, Thrasher, AJ, Mazzolari, E *et al.* (2008). Long-term outcome following hematopoietic stem-cell transplantation in Wiskott-Aldrich syndrome: collaborative study of the European Society for Immunodeficiencies and European Group for Blood and Marrow Transplantation. *Blood* **111**: 439–445.
20. Aiuti, A, Slavin, S, Aker, M, Ficara, F, Deola, S, Mortellaro, A *et al.* (2002). Correction of ADA-SCID by Stem Cell Gene Therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–2413.
21. Hacein-Bey-Abina, S, Le Deist, F, Carlier, F, Bouneaud, C, Hue, C, De Villartay, J *et al.* (2002). Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* **346**: 1185–1193.
22. Sokolic, R, Kesserwan, C and Candotti, F (2008). Recent advances in gene therapy for severe congenital immunodeficiency diseases. *Curr Opin Hematol* **15**: 375–380.
23. Santoni de Sio, FR, Cascio, P, Zingale, A, Gasparini, M and Naldini, L (2006). Proteasome activity restricts lentiviral gene transfer into hematopoietic stem cells and is down-regulated by cytokines that enhance transduction. *Blood* **107**: 4257–4265.
24. Montini, E, Cesana, D, Schmidt, M, Sanvito, F, Ponzoni, M, Bartholomae, C *et al.* (2006). Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* **24**: 687–696.
25. Charrier, S, Dupre, L, Scaramuzza, S, Jeanson-Leh, L, Blundell, MP, Danos, O *et al.* (2007). Lentiviral vectors targeting WASp expression to hematopoietic cells, efficiently transduce and correct cells from WAS patients. *Gene Ther* **14**: 415–428.
26. Dupre, L, Marangoni, F, Scaramuzza, S, Trifari, S, Hernandez, RJ, Aiuti, A *et al.* (2006). Efficacy of gene therapy for Wiskott-Aldrich syndrome using a WAS promoter/cDNA-containing lentiviral vector and nonlethal irradiation. *Hum Gene Ther* **17**: 303–313.
27. Brown, MP, Topham, DJ, Sangster, MY, Zhao, J, Flynn, KJ, Surman, SL *et al.* (1998). Thymic lymphoproliferative disease after successful correction of CD40 ligand deficiency by gene transfer in mice. *Nat Med* **4**: 1253–1260.
28. Woods, NB, Bottero, V, Schmidt, M, von Kalle, C and Verma, IM (2006). Gene therapy: therapeutic gene causing lymphoma. *Nature* **440**: 1123.
29. Li, Z, Dullmann, J, Schiedlmeier, B, Schmidt, M, von Kalle, C, Meyer, J *et al.* (2002). Murine leukemia induced by retroviral gene marking. *Science* **296**: 497.
30. Morales-Tirado, V, Johannson, S, Hanson, E, Howell, A, Zhang, J, Siminovitch, K *et al.* (2004). Selective requirement for the Wiskott-Aldrich syndrome protein in cytokine, but not chemokine, secretion by CD4+ T cells. *J Immunol* **173**: 726–730.
31. Strom, T, Turner, S, Andreansky, S, Liu, H, Doherty, P, Srivastava, D *et al.* (2003). Defects in T-cell-mediated immunity to influenza virus in murine Wiskott-Aldrich Syndrome are corrected by oncoretroviral vector-mediated gene transfer into repopulating hematopoietic cells. *Blood* **102**: 3108–3116.
32. Charrier, S, Stockholm, D, Seye, K, Opolon, P, Taveau, M, Gross, DA *et al.* (2005). A lentiviral vector encoding the human Wiskott-Aldrich syndrome protein corrects immune and cytoskeletal defects in WASP knockout mice. *Gene Ther* **12**: 597–606.
33. Klein, C, Nguyen, D, Liu, C, Mizoguchi, A, Bhan, A, Miki, H *et al.* (2003). Gene therapy for Wiskott-Aldrich syndrome: rescue of T-cell signaling and amelioration of colitis upon transplantation of retrovirally transduced hematopoietic stem cells in mice. *Blood* **101**: 2159–2166.
34. Petrella, A, Doti, I, Agosti, V, Ciarrusso, P, Vitale, D, Bond, H *et al.* (1998). A 5′ regulatory sequence containing two Ets motifs controls the expression of the Wiskott-Aldrich Syndrome protein (WASP) gene in human hematopoietic cells. *Blood* **91**: 4554–4560.
35. Martin, F, Toscano, MG, Blundell, M, Frecha, C, Srivastava, GK, Santamaria, M *et al.* (2005). Lentiviral vectors transcriptionally targeted to hematopoietic cells by WASP gene proximal promoter sequences. *Gene Ther* **12**: 715–723.
36. Toscano, MG, Frecha, C, Benabdellah, K, Cobo, M, Blundell, M, Thrasher, AJ *et al.* (2008). Hematopoietic-specific lentiviral vectors circumvent cellular toxicity due to ectopic expression of Wiskott-Aldrich syndrome protein. *Hum Gene Ther* **19**: 179–197.
37. de la Fuente, MA, Sasahara, Y, Calamito, M, Anton, IM, Elkhal, A, Gallego, MD *et al.* (2007). WIP is a chaperone for Wiskott-Aldrich syndrome protein (WASP). *Proc Natl Acad Sci USA* **104**: 926–931.
38. Kang, HJ, Shin, HY, Ko, SH, Park, JA, Kim, EK, Rhim, JW *et al.* (2008). Unrelated bone marrow transplantation with a reduced toxicity myeloablative conditioning regimen in Wiskott-Aldrich syndrome. *J Korean Med Sci* **23**: 146–148.
39. Westerberg, LS, de la Fuente, MA, Wermeling, F, Ochs, HD, Karlsson, MC, Snapper, SB *et al.* (2008). WASP confers selective advantage for specific hematopoietic cell populations and serves a unique role in marginal zone B-cell homeostasis and function. *Blood* **112**: 4139–4147.
40. Yamaguchi, K, Ariga, T, Yamada, M, Nelson, DL, Kobayashi, R, Kobayashi, C *et al.* (2002). Mixed chimera status of 12 patients with Wiskott-Aldrich syndrome (WAS) after hematopoietic stem cell transplantation: evaluation by flow cytometric analysis of intracellular WAS protein expression. *Blood* **100**: 1208–1214.
41. Stewart, DM, Candotti, F and Nelson, DL (2007). The phenomenon of spontaneous genetic reversions in the Wiskott-Aldrich syndrome: a report of the workshop of the ESID Genetics Working Party at the XIIth Meeting of the European Society for Immunodeficiencies (ESID). Budapest, Hungary October 4–7, 2006. *J Clin Immunol* **27**: 634–639.

42. Yamada, M, Ariga, T, Kawamura, N, Yamaguchi, K, Ohtsu, M, Nelson, DL *et al.* (2000). Determination of carrier status for the Wiskott-Aldrich syndrome by flow cytometric analysis of Wiskott-Aldrich syndrome protein expression in peripheral blood mononuclear cells. *J Immunol* **165**: 1119–1122.
43. Rawlings, SL, Crooks, GM, Bockstoce, D, Barsky, LW, Parkman, R and Weinberg, KI (1999). Spontaneous apoptosis in lymphocytes from patients with Wiskott-Aldrich syndrome: correlation of accelerated cell death and attenuated bcl-2 expression. *Blood* **94**: 3872–3882.
44. Zanta-Boussif, MA, Charrier, S, Brice-Ouzet, A, Martin, S, Opolon, P, Thrasher, AJ *et al.* (2009). Validation of a mutated PRE sequence allowing high and sustained transgene expression while abrogating WHV-X protein synthesis: application to the gene therapy of WAS. *Gene Ther*, in press.
45. Imai, K, Morio, T, Zhu, Y, Jin, Y, Itoh, S, Kajiwara, M *et al.* (2004). Clinical course of patients with WASP gene mutations. *Blood* **103**: 456–464.
46. Blundell, MP, Bouma, G, Calle, Y, Jones, GE, Kinnon, C and Thrasher, AJ (2008). Improvement of migratory defects in a murine model of Wiskott-Aldrich syndrome gene therapy. *Mol Ther* **16**: 836–844.
47. Frecha, C, Toscano, MG, Costa, C, Saez-Lara, MJ, Cosset, FL, Verhoeyen, E *et al.* (2008). Improved lentiviral vectors for Wiskott-Aldrich syndrome gene therapy mimic endogenous expression profiles throughout haematopoiesis. *Gene Ther* **15**: 930–941.
48. Will, E, Bailey, J, Schuesler, T, Modlich, U, Balcik, B, Burzynski, B *et al.* (2007). Importance of murine study design for testing toxicity of retroviral vectors in support of phase I trials. *Mol Ther* **15**: 782–791.
49. Moulding, DA, Blundell, MP, Spiller, DG, White, MR, Cory, GO, Calle, Y *et al.* (2007). Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med* **204**: 2213–2224.
50. Kingsman, SM, Mitrophanous, K and Olsen, JC (2005). Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). *Gene Ther* **12**: 3–4.