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The actin-nucleator promoting factor WASp regulates endo-lysosomal maturation and Toll-like receptor 9 signaling in Dendritic Cells.

Settore scientifico-disciplinare: Biologia Molecolare

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Contents

Abstract

Toll-like receptors (TLRs) are innate immune receptors, which play a key role in both innate and adaptive immune responses. TLRs are important in the recognition of pathogenic components and in triggering inflammatory responses that comprehend various biological mechanisms such as recruitment of inflammatory cells, cytokine production, or activation of adaptive immunity. However, TLRs could be inappropriately activated by self-proteins and endogenous nucleic acids leading to unwarranted inflammation with dangerous outcomes, including autoimmune diseases. Intracellular compartmentalization of TLRs is essential to regulate initiation and termination of signalling, thereby avoiding excessive inflammation. Therefore, studies of mechanisms and adaptors that limit unrestrained activation of innate immune cells upon TLRs triggering are of increasing interest as their potential in understanding autoimmunity.

Wiskott-Aldrich Syndrome (WAS) is a X-linked primary immune deficiency, caused by mutations on WAS-protein (WASp), a hematopoietic specific actin nucleator promoting factor (NPFs). WAS is characterized by recurrent infections, and a marked predisposition to develop autoimmune phenomena. Autoimmune complications occur in 40-72% of patients and the most common autoimmune features include hemolytic anemia, vasculitis, renal disease, arthritis. A general impairment of hematopoietic cell functions contributes to the pathogenesis of the disease since neutrophils, B cells, T cells and DCs deficient for WASp show impaired homing ability, a cellular function that strictly depends on spatio-temporal regulation of actin polymerization. In addition, our previous work has shown that enhanced type-I interferon production is an important feature of WASp deficient DCs.

The mechanisms of immune deficiency in WAS are largely understood whereas the exact cellular mechanisms that link actin alterations to autoimmunity remains to be fully elucidated. In particular, the role of actin and actin-regulatory proteins in coordinating TLRs trafficking across the endosomal system has not yet been investigated.

This thesis aimed to dissect the molecular mechanism that leads to excessive production of type-I interferon in the context of WAS, using a model of WASp null dendritic cells (WKO DCs).

In the first part of this work, we developed and validated new cellular models to study WASp. We demonstrated that, after TLR9 stimulation, WKO cells produce higher amount of type-I IFN and proinflammatory cytokines, both in BM-DCs and Hoxb8-DCs models. Moreover, our dose-response assay of endogenous ligands (immune complex, IC) showed that WKO display a lower TLR9 activation threshold compared to wild-type (WT) DCs.

Intriguingly, our morphological analysis revealed structural alterations in endo-lysosomes of WKO DCs. Using flow cytometry and endocytic probes we observed that the lack of WASp leads to aberration in cargo recycling and degradation.

We then investigated whether WKO structural and functional remodeling could influence TLR9-ligand intracellular trafficking. By blocking protein synthesis, we demonstrated that WASp expression is important to control TLR9 degradation rate. In addition, biochemical and immunofluorescence analysis revealed that exogenous and endogenous TLR9 ligands are engulfed in WKO cells. In particular, ligands traffic slowly across the first endocytic organelles and are degraded less efficiently than in WT DCs.

Finally, using actin inhibitors, we observed that treated cells display impaired endo-lysosome maturation and reduced TLR9 degradation. Most importantly, TLR9 signalling is enhanced leading to higher production of type-I IFN. Taken together, these results prove that the perturbation of actin dynamics closely resembles WASp deficiency phenotype.

In conclusion, our findings shed light on the mechanism underlying excessive TLR9 activation: actin nucleation mediated by WASp is required to maintain a correct endo-lysosomal organization and to control TLR9 threshold, activation and signalling in DCs.

1. Introduction

1.1 The immune system

The immune system is an interactive network evolved under selective pressure imposed by pathogens. As a result, all multi-cellular organisms have developed different defense mechanism to recognize and to combat invading pathogens. A wrong functioning of this system results in under-activity or over-activity leading to severe infections, tumours, allergic and autoimmune diseases. In mammalians, the immune system is divided in two branches, determined by the speed and specificity of the reaction. Innate and adaptive immunity cooperate and are highly interconnected to effectively fight infections and pathogens using different strategies. Cells from both branches are generated in bone marrow by the differentiation of hematopoietic stem cells (HSCs).

Innate immunity represents the first line of defense. This line of defense is triggered by the engagement of pattern recognition receptors (PRRs), receptors that recognize certain molecular structure unique for infectious microbes. On the other hand adaptive immunity, found only in vertebrates, is the specific response using receptors clonally expressed on B and T cells (Medzhitov and Janeway, 1997; Cooper and Alder, 2006). PRRs are strategically expressed at the plasma-membrane, within the cytosol or at the surface of intracellular vesicles of specific cells that are the first to encounter pathogen during infection. Among these cells there are the antigen presenting cells (APCs), such as dendritc cells (DCs).

PRRs are able to recognize two different types of signals: exogenous and endogenous. Exogenous molecules from bacteria or viruses are called specific pathogen-associated molecular patterns (PAMPs), DNA or ATP are endogenous signals called damage-associated molecular patterns (DAMPs) from damaged or dead cells. The direct recognition by PRRs of PAMPs results in endosomal and cytoskeletal dynamics and induces the expression of innate response genes: pro-inflammatory (cytokines and chemockines) and co-stimulatory molecules. Innate immunity with this initial inflammatory response leads to the activation of

adaptive immunity by recruiting and activating B and T cells that are instructed to limit pathogen spread and eradicate the invaders.

In the last years several classes of PRRs have been discovered and characterized in detail: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), leucine-rich repeat–containing receptors (NLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and AIM-2 like receptors, as well as OAS proteins and cGAS; a family of enzymes that function as intracellular sensors of nucleic acids (Pichlmair and Sousa, 2007; Iwasaki and Medzhitov, 2015).

1.1.1 Toll-like receptors (TLRs)

TLRs family is the major class of PRRs and best characterized in mammalian species. Although the exact gene number is different between species, for example ten TLRs have been identified in humans and twelve in mice. TLR1 to TLR9 are conserved in both species. TLRs are type I transmembrane proteins composed of extracellular ectodomain which contain leucin-rich repeats (LRRs) involved in protein-protein interaction that recognize PAMPs. TLRs contain a single transmembrane αhelic and a conserved cytosolic domain containing a Toll/IL-1 receptor (TIR) domain that initiate downstream signaling. Expression of these receptors is mostly restricted to endothelial, epithelial and immune cells such as DCs and macrophages. TLRs are divided in two different groups depending on their localization in the cells and the respective PAMPs ligand. One group is composed of TLRs expressed on the cell surface such as TLR1, TLR2, TLR4, TLR5 TLR6 and TLR11 in mice. These TLRs generally recognize and bind microbial membrane and molecules found on the surface of pathogens such as lipid, protein and lipoprotein. The second group is composed of intracellular TLRs: TLR3 TLR7 TRL8 TRL9 expressed inside the cells on endosomal or lysosomal vesicles or endopolasmatic reticulum (ER). These receptors in general bind structures that are accessible upon uptake and destruction of pathogens such as nucleic acid (Iwasaki and Medzhitov, 2004; Pelka *et al.*, 2016).

Each TLR differs from the others in ligand specificities, expression patterns and in the target genes that they can induce. Among cell surface TLRs, TLR4 is important to recognize bacterial lipopolysaccharide (LPS); TLR2, TLR1 and TLR6 plays critical role in recognition

of a wide variety of PAMPs including lipoproteins, peptidoglycans of bacteria, mannan, zymosan from fungi, lipotechoic acids and tGPI-mucin from parasites (*Trypanosoma cruzi*). TLR5 is involved in bacterial flagellin recognition and TLR11 recognize apicomplexan profilins. TLRs have an important role in the antiviral immune response thanks to their ability to induce type I interferons (Type-I-IFNs): a family of cytokines specialized to induce and to coordinate immunity to viral infections. For instance, TLR3 recognizes viral double-stranded RNA (dsRNA), its synthetic analog polyinosinic acid-cytidylic acid (poly(I)•poly(C)) and self RNAs derived from damaged cells. TLR9 bind bacterial and viral DNA rich in unmethylated-CpG (cytidine-phosphate-guanosine) DNA motifs and its synthetic analogous (CpG-containing phosphorothioate modified oligodeoxynucleotides) CpG-ODN. TLR7 recognizes single-stranded (ss)RNA from virus in plasmacytoid DCs (pDCs) and RNA from streptococcus B bacteria in conventional DCs (cDCs) (Kawai and Akira, 2006; Kawasaki and Kawai, 2014).

1.1.2 TLR trafficking

Although sharing common functional and structural features, individual TLRs possess exclusive characteristics, including the sub cellular compartments to which they localize. TLRs 1, 2, 4, 5, and 6 localize to the plasma membrane, whereas TLRs 3, 7, 8, and 9 localize within intracellular compartments such as endosomes and lysosomes. The localization of TLRs within intracellular compartments places these receptors in a strategic position to encounter nucleic acids released from engulfed microbes and are also necessary to provide a mechanism to prevent recognition of self nucleic acids, for function and activation of this subset of receptors. For example, the pH of endosomes and lysosomes is very important to activate TLRs and neutralizing it with drugs such as bafilomycin A1 and chloroquine inhibits their activation (Rutz *et al.*, 2004). Furthermore, initiation of anti-viral responses by TLRs is generally reserved for TLRs activated within endosomes where they activate transcription factors IRF1, IRF3 or IRF7 to induce type I IFN (Kagan et al. 2010).

<u>Trafficking from the ER</u>
While it is well understood that correct trafficking of nucleic acid sensing TLRs is a critical regulatory step for activation and discrimination between self and non-self, our knowledge of

how these receptors get access to signaling compartments remains still incomplete and under investigation. A critical step of regulation may be at the initial export from ER. Alterations at this point may determine the level of functional receptor present in endosomes and lysosomes and thus influencing the threshold of receptor activation. In autoimmunity it is highly relevant to control this threshold, such as over expression of TLR7 is sufficient to cause systemic lupus erythematosus (SLE) in mice (Barton, Kagan and Medzhitov, 2006; Pisitkun *et al.*, 2006; Subramanian *et al.*, 2006; Pawar *et al.*, 2007). One common cofactor that controls TLR trafficking and exit from the ER is a polytopic transmembrane protein UNC93B1. *Unc93b1* was originally identified in a mouse mutagenesis screen where a point mutation (H412R) resulted in a defect of TLR3, 7, and 9 responses but not in surface localized TLRs (Tabeta *et al.*, 2006; Kim *et al.*, 2008). Additional studies demonstrated that this defect was due to a failure of TLRs to reach their endolysosomal compartments. In these mice, the mutated UNC93B1 is unable to cooperate with TLRs and both UNC93B1 and TLRs remain restricted to the ER (Lee *et al.*, 2013; Pelka *et al.*, 2016). Based on these observations UNC93B1 acts as a trafficking chaperone guiding TLRs to the different sub-cellular compartment in which signaling occurs. Furthermore ER resident proteins are required for proper trafficking of multiple TLRs, UNC93B1 facilitates incorporation of TLR9 into COPII vesicles, which transport protein cargo from the ER to Golgi (Lee *et al.*, 2013). UNC93B1is also clinical relevant because was found that UNC93B1 deficiency in humans predisposes healthy individuals to herpes simplex virus (HSV) encephalitis (Casrouge *et al.*, 2006). In addition, another adaptor was identified as a protein that co-immunoprecipitates with TLRs: PRAT4A (Wakabayashi *et al.*, 2006). PRAT4A is a ubiquitous and highly conserved soluble 276 amino acid ER luminal protein. Macrophages, bone marrow-derived DCs (BMDCs) and B cells isolated from *Prat4a*$^{-/-}$ mice showed reduced cytokine production in response to ligands for TLR1, TLR2, TLR4, TLR7 and TLR9, but not for TLR3 (Takahashi *et al.*, 2007). Knockdown of *Prat4a* mediated by short hairpin RNA (shRNA) in B cell lines obstructed passage of TLR1 and TLR4 through the Golgi and prevented ligand-induced trafficking of TLR9 from the ER to endolysosomes (Takahashi *et al.*, 2007).

Trafficking beyond the ER

Upon ER exit, TLRs are sorted to endosomal compartments passing through the Golgi (Ewald *et al.*, 2008, 2011; Chockalingam *et al.*, 2009). Although recent studies have identified some of the key players, the molecular mechanisms underlying these sorting events are poorly

understood. After ER exit UNC93B1 remains associated with TLRs and plays a role in these later trafficking events (Kim *et al.*, 2008; Lee *et al.*, 2013). It was shown that endosomal trafficking of UNC93B1 depends on a tyrosine-based motif near its C-terminus that can act as protein sorting signals by binding to AP complexes including AP-1, 2, and 3. These AP complexes are important in different pathway: AP-1 is involved in trafficking pathways from the TGN to endosomes, AP-2 has a role in clathrin-mediated endocytosis and AP-3 mediates the trafficking of proteins to lysosomes or lysosome-related organelles (LROs) (Braulke and Bonifacino, 2009). Binding between UNC93B1 by tyrosine-based motif and AP complexes is stronger with AP-2 and lesser to AP-1, suggesting that UNC93B1 might traffic via both the route, direct and the indirect, to endolysosomal compartments. Mutation on tyrosine-based motif caused aberrant trafficking of UNC93B1 and had thoughtful effects on TLR responses (Pelka *et al.*, 2016). For example in cells expressing UNC93B1 mutant, TLR9 is not able to reach endosomes and accumulates at the plasma membrane (Lee *et al.*, 2013). This remarkable finding indicates that TLR9 traffics via the cell surface end route to endosomes, requiring a proteolytic processing as a mechanism to prevent activation at the cell surface. Unlike TLRs localized on the plasma membrane the ectodomains of TLRs 3, 7, and 9 are proteolytically processed by endosomal proteases such as cathepsins and asparagine endopeptidase (Ewald *et al.*, 2008). This event separates an N-terminal portion from the rest of the TLR. The C-terminal fragment contains the part of truncated ectodomain, transmembrane, and cytosolic regions and acts as a functional, cleaved receptor. This proteolytic event is not required for ligand binding, but it is essential to initiate downstream signals. Inhibition of this process blocks receptor signaling (Lee and Barton, 2014).

1.1.3 TLR signaling

Upon recognition of their different ligands, TLRs recruit members of a set of TIR domain-containing adaptors to start a signalling pathway to activate NF-κB, MAPKs and to induce expression of variety of host defence genes such as inflammatory cytokines, chemokines and type I IFN. There are four adaptor molecules, namely Myeloid differentiation primary response 88 (MyD88), TIR-associated protein (TIRAP), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Akira, Uematsu and Takeuchi, 2006); and two different well characterized pathways. MyD88

dependent pathway and TRIF dependent pathway, leading to the production of proinflammatory cytokines and type I IFNs, respectively *(Fig 1.1)*.
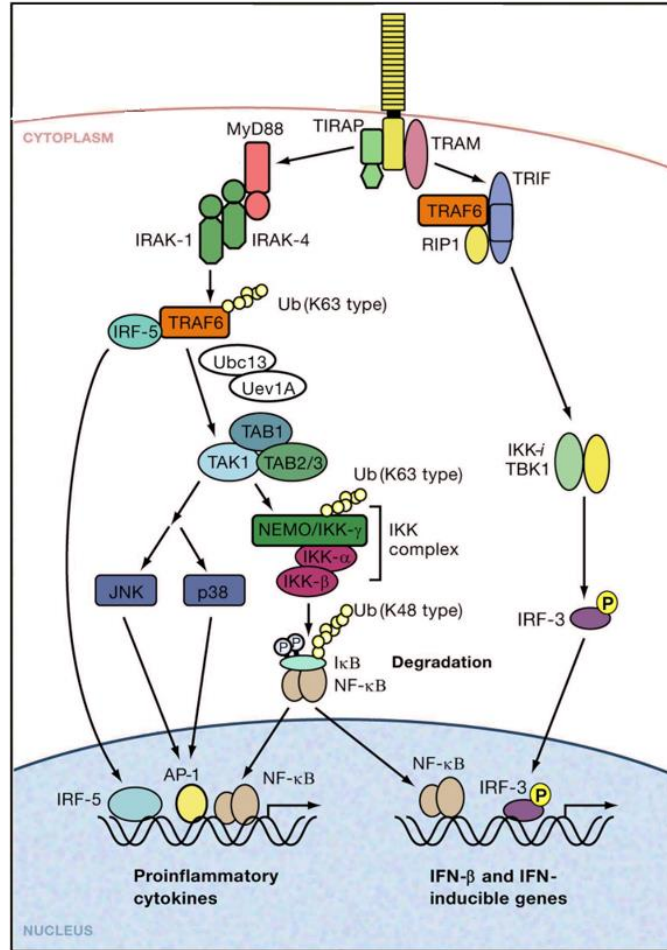
Figure 1.1 The TLR signaling pathway. TLR stimulation recruits TIR-domain-containing adaptors including MyD88 and TIRAP to the receptor, and the subsequent formation of signaling complex. MyD88 activation recruit IRAK4-IRAK1 that activates TRAF6. TRAF6 acts as an E3 ubiquitin ligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and UEV1A. This ubiquitination activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IkB undergoes to ubiquitination and degradation. Free NF-kB translocate into the nucleus and initiates the expression of proinflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TRIF-dependent signaling pathway via TRAM induce type I IFNs. TRIF activates IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs.

Modified from Akira et al. 2006 *Cell*

MyD88

MyD88 is used by all TLRs except TLR3. Upon stimulation, MyD88 associates with the cytoplasmic portion of TLRs and then recruits IL-1R-associated kinase 4 (IRAK-4), IRAK-1

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and forms with them a complex called Myddosome. In TLR2 and TLR4 signalling, another adaptor, TIRAP, is required for recruiting MyD88 to the receptor (Fitzgerald, 2001; Horng and Medzhitov, 2001; Akira, Uematsu and Takeuchi, 2006) During myddosome formation IRAK4 activates IRAK1 by phosphorylation, which is then auto-phosphorylated at several sites and released from MyD88 to allow association with TNFR-associated factor 6 (TRAF6). TRAF6, acts as an ubiquitin protein ligase (E3) (Li *et al.*, 2002), together with a ubiquitination E2 enzyme complex (UBC13 and UEV1A), catalyses the formation of a complex with TAK1, TAB1 TAB2/3 that activates TAK1(Wang *et al.*, 2001). This complex induces the formation of a K63-linked polyubiquitin chain on IKK-γ/NF-kB essential modulator (NEMO) which modulates the e activation of NF-kB and MAP kinases, resulting in induction of genes involved in inflammatory responses. Moreover, TRAF6 activate IRF5 that translocate into the nucleus and regulates the expression of cytokine genes (Takaoka *et al.*, 2005)

TRIF

TRIF adaptor is important to induce type I IFN production in addition to proinflammatory signals after TLR3, TLR4, TLR7, and TLR9 stimulation. TRAM acts as a connecting adaptor between TRIF and TLR3 and TLR4. TRIF interact with TRAF6 and receptor-interacting protein 1 (RIP1) and activate TBK1-IKK-I complex which mediate the phosphorylation and the translocation into the nucleus of IFN-regulatory factor 3 (IRF3). IRF3 and bind to the ISREs, resulting in the expression of a set of IFN-inducible genes. Also TLR7 and 9 elicit type-I IFN induction, through MyD88, in a pathway that involves the kinases IRAK1 and IRAK4 and the IFN-regulatory factor 7 (IRF-7) (Akira, Uematsu and Takeuchi, 2006; Patel *et al.*, 2012; Kawasaki and Kawai, 2014).

All together these signaling cascade induce a variety of genes encoding for type-I IFN, inflammatory cytokines (IL-12, TNF and IL-6) co-stimulatory molecules (CD40, CD86 and CD80) and chemokines receptors (CCR2, CCR5 and CCR7) (Akira, Takeda and Kaisho, 2001). The discovery of TLRs and the identification of these receptors as modulators in immune system has been an important advancement in the field of immunology. Hoffman and Beutler first described TLRs in fruit flies, instead Ralph Steinman discovered dendritic cell as

the cell type that utilizes TLRs to initiate adaptive immunity (Maglione, Simchoni and Cunningham-Rundles, 2015).

1.1.4 TLR negative regulation

TLRs activation is essential to induce the innate immune response and to enhance adaptive immunity against pathogens. However, excessive TLR activation is a double-edged sword. Sustained inflammatory signalling or activation by endogenous molecules can disturb immune homeostasis and members of the TLR family are involved in the pathogenesis of immunodeficiency, autoimmunity and inflammatory diseases like rheumatoid arthritis, chronic obstructive pulmonary disease, chronic enterocolitis, diabetes, asthma, cardiomyopathy, systemic lupus erythematosus and atherosclerosis (*Table 1.1*) (Cook, Pisetsky and Schwartz, 2004; Lang and Mansell, 2007)

TLR	PAMPs	DAMPs	Disease
TLR1	(w/TLR2) triacyl lipoprotein	n.d.	
TLR2	Lipoproteins (w/TLR1) triacyl lipoprotein (w/TLR6) diacyl lipoprotein, LTA, zymosan	(w/TLR6) HMGB1, HSPs, ECM	Candidiasis
TLR3	dsRNA	mRNA	WNV
TLR4	LPS, viral envelop proteins	HMGB1, HSPs, ECM, Ox-phospholipids, β-defensin 2 (w/TLR6) Amyloid-β, Ox-LDL	Sepsis, EAE, Atherosclerosis, COPD, Asthma
TLR5	Flagellin	n.d.	
TLR6	(w/TLR2) Diacyl lipoprotein, LTA, Zymosan	(w/TLR2) HMGB1, HSPs, ECM	
mTLR7/hTLR8	ssRNA	ssRNA (immune complex)	
TLR9	DNA, hemozoin	DNA (immune complex)	Malaria, SLE
TLR10	Unknown	n.d.	
TLR11	Profilin-like molecule Uropathogenic bacteria	n.d.	

Table 1.1 TLRs ligands and related diseases. COPD, chronic obstructive pulmonary disease; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; HMGB1, high mobility group; HSPs, heat shock proteins; LTA, lipoteichoic acid; n.d., not determined; Ox-LDL, oxidized low-density lipoprotein; SLE, systemic lupus erythematosus; WNV, West Nile virus.

from Akira et al. 2012 *Trends in Immunology*

Therefore, the strength and duration of TLRs responses must be strongly controlled, to maintain homeostasis and to avoid inappropriate inflammatory responses, TLRs signaling is negatively controlled by multiple mechanisms (*Table1.2*).

Regulator	Expression and induction	Affected TLR	Possible mechanism
sTLR2	Constitutively expressed in breast milk and plasma	TLR2	TLR2 antagonist
sTLR4	ND	TLR4	Blocks interaction of TLR4 and MD2
MyD88s	LPS-induced expression, mainly in spleen	TLR4	MyD88 antagonist
IRAKM	LPS-induced expression by monocytes	TLR4,9	Inhibits phosphorylation IRAK1
SOCS1	LPS- and CpG-induced expression by macrophages	TLR4,9	Suppresses IRAK
NOD2	ND	TLR2	Suppresses NF-κB
PI3K	Constitutively expressed by most cells	TLR2,4,9	Inhibits p38, JNK and NF-κB function
TOLLIP	Constitutively expressed in most tissues	TLR2,4	Autophosphorylates IRAK1
A20	LPS-induced expression by macrophages	TLR2,3,4,9	De-ubiquitylates TRAF6
ST2L	LPS-induced expression by macrophages	TLR2,4,9	Sequesters MyD88 and MAL
SIGIRR	Mainly expressed by epithelial cells and immature dendritic cells but downregulated by activation	TLR4,9	Interacts with TRAF6 and IRAK
TRAILR	Constitutively expressed by most cells	TLR2,3,4	Stabilizes IκBα
TRIAD3A	Constitutively expressed by most cells and tissues	TLR4,9	Ubiquitylates TLRs

Table 1.2 Negative regulators of TLRs. IRAK, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; ND, not determined; NF-κB, nuclear factor-κB; NOD2, nucleotide-binding oligomerization domain protein 2; PI3K, phosphatidylinositol 3-kinase; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signalling 1; sTLR, soluble decoy TLR; TOLLIP, Toll-interacting protein; TRAF, tumour-necrosis factor-receptor-associated factor; TRAILR, tumour-necrosis factor-related apoptosis-inducing ligand receptor.

From Liew et al. 2005. *Nature Reviews Immunology*

Extracellular regulation

Soluble decoy TLRs (**sTLRs**) compete with TLR agonists and are highly active in the first line of negative regulation. STLRs directly attenuate TLR signalling and consequently prevent acute inflammatory responses to pathogenic ligands and stress proteins. Although, in mammalian host, there is only a single copy of the TLR4 gene, several mRNA products have been detected (Qureshi *et al.*, 1999) indicating the presence of TLR4 isoforms. It was demonstrated, by screening a mouse macrophage cDNA library, the presence of a soluble form of TLR4 (sTLR4) that inhibit NF-kB activation *in vitro* after LPS stimulation (Iwami *et al.*, 2000). The mechanism by which sTLR4 attenuates TLR4 function is currently unclear. The most obvious explanation would be that sTLR4 blocks the interaction between TLR4 and its co-receptors (MD2 and CD14), leading to signalling termination (Hyakushima *et al.*, 2004). Similarly, six isoforms of soluble TLR2 have been described in human milk and

plasma (LeBouder *et al.*, 2003). sTLR-2 was found to inhibit IL-8 and tumour necrosis factor (TNF) through the direct interaction with CD14 following stimulation with bacterial lipopeptide.(Iwaki *et al.*, 2002)

<u>Transmembrane protein regulator</u>

Transmembrane negative regulators are proteins that inhibit TLR functions by either interfering with the binding of TLR ligands to their specific TLR or sequestering the TLR adaptors. Suppressor of tumorigenicity 2 (ST2), single immunoglobulin IL-1-related protein (SIGIRR), both members of the TIR superfamily, and TNF-related apoptosis-inducing ligand receptor (TRAILR) belong to this class of negative regulators.

ST2 is a type-1 transmembrane receptor and is present in two main forms, ST2L and sST2 and sequesters adaptor proteins within TLR signalling pathways. ST2 acts specifically on the MyD88-dependent pathway since studies using mice deficient in both ST2L and sST2 produced an extensive amount of pro-inflammatory cytokines in response to IL-1 and LPS, bacterial lipopeptide CpG; however, the response to polyI:C (TLR3) was not impaired This observation was also supported co-precipitation experiments showed a direct interaction between ST2L and MyD88, but not TRIF (Brint *et al.*, 2004). Takezako and colleague showed the negative regulatory effect of ST2 protein also on IL-6 IL-1β and TNFα production in a human monocytic leukaemia cell line, THP-1 cells (Takezako *et al.*, 2006).

SIGIRR also known as TIR-8, is highly expressed in epithelial cells and DCs, but not on primary macrophages (Wald *et al.*, 2003). It was shown that the overexpression of SIGIRR in bone marrow-derived DCs leads to the inhibition NF-κB activation consequently the inhibition of IL-1 and -18 production (Polentarutti *et al.*, 2003). Similarly to ST2, SIGIRR acts on the MyD88-dependent pathway since SIGIRR deficient mice display enhanced activation of NF-kB after LPS or CpG DNA stimulation, resulting in exaggerated cytokine production, but not to TLR-3 ligand (Garlanda *et al.*, 2004).

Studies using **TRAILR**-deficient mice showed that they had enhanced cytokine production when macrophages were stimulated with agonist for TLR2,3 and 4, but not TLR9 (Diehl *et al.*, 2004). Furthermore, these mice had significantly greater clearance of mouse cytomegalovirus infection, associated with amplified levels of INF-β, INF-γ and IL-12. TRAILR negative regulate TLRs activation by IkBα stabilization resulting in the decrease of nuclear translocation of NF-kB (Diehl *et al.*, 2004).

<u>Intracellular negative regulators</u>

The next line of defense to negative regulate TLRs activation is through several intracellular negative regulators. This big class of molecules include MyD88s, IRAKM, SOCS1, PI3K, and A20 (*Figure1.2*)

MyD88s is a short-splice MyD88 variant that lacks an intermediate linker sequence present in complete MyD88 and is detected only in the spleen and, less strongly, in the brain (Janssens *et al.*, 2002; Burns *et al.*, 2003) It was reported that MyD88s overexpression, in monocytes stimulated with LPS, resulted in impaired activation of NF-κB as a result of inhibited activation of IRAK439. However, without affecting TNF-induced NF-kB activation (Janssens *et al.*, 2003).

IRAKM, belong to the IRAK family that lack active serine/threonine kinases and its expression is restricted to monocytes/macrophages (Wesche *et al.*, 1999; Janssens and Beyaert, 2003). *In vivo* experiment performed on IRAKM$^{-/-}$ mice showed high inflammatory responses to bacterial ligands and defective LPS tolerance in comparison to control mice (Kobayashi *et al.*, 2002)50. From *in vitro* experiment on HEK293 cells it was demonstrated that IRAKM prevents dissociation of IRAK1/IRAK4 from the MyD88 receptor complex, therefore inhibiting activation of signalling cascade (Kobayashi *et al.*, 2002) 50.

Suppressor of cytokine signalling-1 (**SOCS1)** belong to SOCS family proteins. All members of the SOCS family share a conserved central Src homology (SH)-2 domain and a unique carboxyl motif at the C-terminus named the SOCS box. SOCS activation is induced by cytokines and function as negative-feedback regulators within cytokine signalling (Lang and Mansell, 2007). It has been shown that SOCS1 deficiency in mice is lethal. Mice within 3 we(Marion *et al.*, 2011)eks of birth as a result of multiorgan inflammation due to a hypersensitivity to IFN-γ. These SOCS1$^{-/-}$ mice also produce significantly increased levels of pro-inflammatory cytokines and nitric oxide and upon LPS or CpG DNA stimulation (TLR-4 and TLR9 agonist) (Alexander *et al.*, 1999) Investigations by Mansell and colleagues have shown the mechanism for SOCS1 directly regulating TLR2 and TLR4 mediated signalling. SOCS1 directly target Mal for polyubiquitination and subsequent proteosomal degradation and inhibit p65 phosphorylation and subsequent NF-kB transactivation (Mansell *et al.*, 2006)

A20 is a zinc ring finger protein expressed in numerous cell types, its expression is rapidly increases in response to LPS and TNF, implicating it as a possible regulator of inflammatory responses (Opipari, Boguski and Dixit, 1990; Krikos, Laherty and Dixit, 1992).

Studies carried on A20 deficient mouse macrophages demonstrate that there is a significant increase in proinflammatory cytokines in response to TLR2, TLR3 and TLR9 ligands (Boone *et al.*, 2004). A20, is associated to TNF mediated NF-kB activation and was also identified as a cysteine protease de-ubiquitylating protein able to prevent TLR signalling via TRAF6 (Boone *et al.*, 2004).

PI3K is a heterodimer that consists of a regulatory subunit (p85) and a catalytic chain (p110). It is constitutively expressed by most cells and functions as an early negative regulator of TLR signaling (Katso *et al.*, 2001). Dendritic cells from PI3K-deficient mice show enhanced IL-12 synthesis in response to the TLR4, TLR2 and TLR9 ligands if compare to control cells. Moreover, *in vivo* experiment on PI3K-deficient mice are more resistant than wild-type mice to infection with Leishmania major, which is a prototypic TH2-mediated parasitic disease (Fukao *et al.*, 2002). However, the precise mechanism by which PI3K inhibits TLR signaling is not identified yet.

NOD2 belong to the nucleotide-binding oligomerization domain family. NOD2 contains a C-terminal leucine-rich repeat domain, indicating that might be a pathogen pattern recognition receptor (Harton *et al.*, 2002; Inohara and Nuñez, 2003), and it can recognize the bacterial product muramyl dipeptide (MDP), a derivative of bacterial peptidoglycan (Girardin *et al.*, 2003; Inohara *et al.*, 2003). Experiment on spleen cells from NOD2$^{-/-}$ mice developed an elevated TH1-cell response, which is characterized by IL-12 and IFN-γ production, in response to the TLR2 agonists but not in response to other TLR ligands. Indicating NOD2 as a negative regulator of TLR2 signaling(Watanabe *et al.*, 2004). Patients with Crohn's disease carry a mutation in the leucine-rich repeat region of the NOD2 gene, which results in weakened MDP recognition (Hugot *et al.*, 2001; Ogura *et al.*, 2001). However, two different studies provided evidence that NOD2 might not be a negative regulator of TLR2 signalling (Maeda *et al.*, 2005; Pighin JA1, Zheng H, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst L, 2013).

Toll-interacting protein **TOLLIP** is able to interact with several members of the TIR superfamily members, including TLR2 and TLR4 (Bulut *et al.*, 2001; Zhang and Ghosh, 2002). TOLLIP interact with IRAK1 and its phosphorylation decrease when TOLLIP is overexpressed; and TLR2 and TLR4 mediated NF-κB activation are inhibited. After TLR stimulation, IRAK1 causes phosphorylation of TOLLIP (Zhang and Ghosh, 2002) and is

possible that its phosphorylation facilitates the ubiquitination of IRAK1 and its subsequent degradation (Liew *et al.*, 2005).
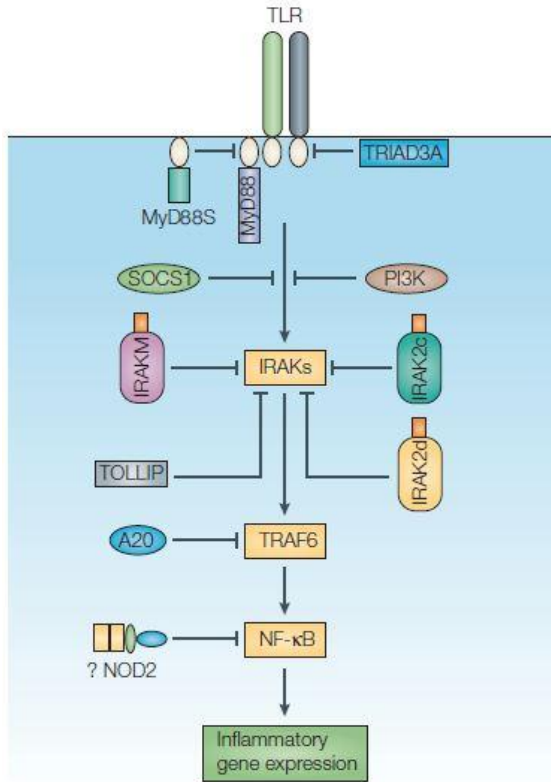

Figure 1.2 Intracellular Toll-like receptor regulators. TLRs signalling pathways are tightly regulated by endogenous regulators at multiple levels. MyD88s antagonizes MyD88 functions. Inhibitory proteins such as SOCS1, IRAKM, TOLLIP, IRAK2c and IRAK2d selectively suppress IRAK function by targeting various stages of the TLR signalling pathways. PI3K negatively regulates some TLR responses through an as-yet-unknown mechanism. A20 deubiquitylates TRAF6 and affects both MyD88-dependent and MyD88-independent pathways. NOD2 might inhibit TLR2 signalling by suppressing nuclear factor-κB (NF-κB) activity. TRIAD3A promotes ubiquitylation and degradation of certain TLRs.

From Liew et al. 2005. *Nature Reviews Immunology*

Reduction of TLRs expression

TLRs signaling can be also controlled by reducing its expression. Expression reduction might be reached by the inhibition of TLRs through anti-inflammatory cytokines or degradation by ubiquitylation. Ubiquitylation is a general mechanism used to regulate cell-membrane receptor by promoting proteolytic degradation of the protein. **TRIAD3A**, (member of the TRIAD3 family of RING-finger E3 ligases) was found to bind the cytoplasmic domain of TLR9 and TLR4 and to promote ubiquitylation and degradation of these receptors (Chuang and Ulevitch, 2004). Overexpression of TRIAD3A markedly reduced TLR4 and TLR9 activation response to LPS and CpG DNA but not polyI:C or bacterial lipopeptide (a TLR2 ligand). On the other hand, *in vitro* knockdown of TRIAD3A knockdown by small interfering RNA (siRNA) led to enhanced TLR expression and increased responses to CpGB and LPS

and CpG(Chuang and Ulevitch, 2004). These results offer evidence that TRIAD3A selectively regulates TLR4 and TLR9 degradation through binding and ubiquitylation. Also, anti-inflammatory cytokines, like transforming growth factor-β (TGF-β) and IL-10 may regulate the expression and functions of TLRs. **TGF-β1** suppresses LPS-mediated function by inhibiting TLR4 expression (McCartney-Francis, Jin and Wahl, 2004), and induces MyD88 degradation by the proteasome, down-regulating TLR signaling (Naiki *et al.*, 2005). In human dendritic cells was reported that **IL-10** induced by TLR2 activation could suppress IL-12 production by TLR3 and TLR4, signaling indicating that crosstalk among TLRs might affect the outcomes of immunity(Re and Strominger, 2004).

TLR localization

Another important step to negative regulate TLRs signalling is their subcellular compartmentalization, but also TLRs ligand localization. It is still a material of discussion where these TLRs are localized in resting cells and which trafficking pathways they use to reach their respective signalling compartments. It was reported that only plasmacytoid DCs (pDCs) are able to respond to CpG-A DNA but not conventional DCs (cDCs). An explanation for this observation was provided by CpG-A DNA compartmentalization. In pDCs CpG-A DNA was retained for long periods in the endosomal vesicles, together with the MyD88 IRF-7 complex. However, in cDCs, was rapidly transferred to lysosomal vesicles. Using cationic lipid, to manipulate the endosomal retention of CpG-A DNA in cDCs, robust TLR9 responses were achieved also in cDCs. Therefore, the spatiotemporal regulation of TLR9 ligand seems to be a determining factor in the responsiveness of pDCs and DCs to CpG-A DNA (C. Honda, K. Ohba, Y. Hideyuki, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, 2005a).

An emerging theme is that the strictly regulated subcellular location of TLRs controls the access of the TLRs to their agonists and therefore regulates responses. Recent evidence implicates the actin cytoskeleton as a key regulator of cell signalling. The cellular cytoskeleton is a track for vesicle and organelle movement and is an incessantly remodelled dynamic network that provides force and support to cellular structures (Clainche and Carlier, 2008; Trimble and Grinstein, 2015; Mattila, Batista and Treanor, 2016). It will be fascinating to learn more about the molecular mechanism underlying such controls and if any of these provide targets for novel therapeutics to regulate TLR activity in allergy and autoimmunity.

1.1.5 TLRs and autoimmunity

Autoimmunity arise by several mechanisms that relate to the presence of auto-reactive immune cell subsets and loss of immunological tolerance (Anders *et al.*, 2005). Loss of tolerance may lead to uncontrolled activation of self-reactive reactive B and T cells which induce autoimmunity assisted by the cells of the innate immunity (DCs). TLR expression has been found in various types of immune cells, including DCs, monocytes, macrophages, neutrophils, eosinophils, mast cells, epithelial cells T cells and B cells, (Liu and Zhao, 2007; Hosseini *et al.*, 2015) considering TLRs as a link between the innate and the adaptive immune responses. In several autoimmune pathologies an association to dysregulated or, continuous TLRs activation has been associated to pathogenesis of autoimmunity (Abdollahi-Roodsaz *et al.*, 2007).

Systemic Lupus Erythematosus (SLE)

Some years ago, Pascual group noted an increased level of IFN-α in the serum of lupus patients (Hooks et al., 1979) that was later confirmed in a number of studies. Even if other cytokines are also increased in the serum of these patients, IFN levels best coincide with exacerbation of the disease. It has been shown that IFN-α treatment for a variety of conditions, like chronic viral infections and cancer, also induce autoimmune manifestations like lupus. Furthermore, evidence for a role of type-I IFN in SLE pathogenesis was yielded by the discover that sera of lupus patients, induced in vitro maturation of normal peripheral blood monocytes into DCs capable of efficiently capturing and presenting antigens, and the active factor for this effect was IFN-α (Blanco, Palucka and Gill, 2001). In addition, studies on peripheral blood mononuclear cells of lupus patients exhibited increased expression of IFN-α-induced genes (ISGs) that associated with disease severity in both early and late stages of the disease (Baechler *et al.*, 2003; Bennett *et al.*, 2003; Kirou *et al.*, 2004; Peterson *et al.*, 2004). Studies performed on animal models of spontaneous lupus have made it possible to directly demonstrate the role of IFN-α/β in this disease. Theofilopoulos's group showed a significant amelioration of lupus disease in IFNAR1-deficient mice (Santiago-Raber et al., 2003). Lack of *Ifnar1* gene (homozygous or heterozygous) led to reduction of many disease manifestations, including hemolytic anemia, glomerulonephritis, levels of IgG anti-dsDNA autoantibodies, and mortality. The lupus-promoting effects arbitrated by type-I IFN involved

many immunocyte subsets. In IFN-α/β-deficient mice were observed a decrease in numbers of B1 and conventional B2 subsets, spontaneous antibody and autoantibody-secreting (Milner *et al.*, 2005), the number of T cells and glomerular immune complex deposits and lymphadenopathy in lupus prone mice knock-out for the *Ifnar1* gene (Braun, Geraldes and Demengeot, 2003). Overall, type-I IFN signalling seems to be crucial in switching on pathways that lead to pathogenicity in lupus-predisposed backgrounds. However, it has not well-known whether this switch is turned on in lupus because of a primary defect, such as hyper-responsiveness to stimuli, or because of increased levels of apoptotic cells and autoantigen-autoantibody immune complexes. However, it has been demonstrated that in SLE patients pDCs secrete IFN-α/β in response to DNA/RNA/immunocomplexes deriving from apoptotic/necrotic material and chronically stimulate TLR7 and TLR9 (Rönnblom, Eloranta and Alm, 2006). In addition, Gilliet's group showed that neutrophil extracellular traps (NETs), complexes composed of antimicrobial peptides and self-DNA produced by activated neutrophils, also play a critical role in pDC activation by chronic triggering of TLR9 (Lande *et al.*, 2012). Based on these evidences it has been proposed that induction of IFN-α/β is a central event in lupus-associated systemic autoimmunity. In this model initial phase is mediated by uptake of IC by pDCs and increased type-I IFN production cause cDC activation, leading to high expression of MHC-I and co-stimulatory molecules. Production of inflammatory cytokines like IL-6, IL-10 and IL-15 by cDCs, leads B cell differentiation to plasma cells and autoantibody production. Autoantibodies and/or immune complexes bind to cell surfaces and tissue and cause systemic autoimmune disease.

Rheumatoid Arthritis (RA)

RA affects ~1% of the population causing chronic inflammation leading to the destruction of articular tissues. The exact molecular pathogenesis of the disease remains unclear. However, TLRs are highly expressed in patients with RA. Increased expression of TLR2 and TLR4 on monocytes and peripheral blood from patients with RA has been demonstrated (Abdollahi-Roodsaz et al., 2007). Moreover, some endogenous TLR ligands can be found in arthritic joints. TLR3 can be activated by RNA released from necrotic synovial fluid cells of RA patients (Brentano et al., 2005). In rheumatoid synovium was shown the presence of endogenous TLR4 ligands such as fibronectin fragments and heat-shock proteins (HSPs) (Scott, Delamere and Walton, 1981; Roelofs et al., 2006). In addition, TLR4 might be activate by serum and synovial fluid of RA patients suggesting the presence of TLR4–activating substances in RA serum and joints.

Multiple Sclerosis (MS)

In MS, DCs, NK, B and T cells, migrate to the central nervous system (CNS) and cause myelin destruction, axon damage and neuronal cell death (Waldner, Collins and Kuchroo, 2004) TLR2 and TLR4 of DCs recognized endogenous ligands, such as high mobility group box 1 (HMGB1), and lead to the production of IL1, IL6 and IL12. These cytokines stimulate differentiation of naive T cells into Th1 and Th17 cells and INFγ and IL17 secretion. INFγ/IL17-producing T cells facilitate leukocyte migration through the blood-brain barrier and contribute to CNS damage (Hosseini et al., 2015).

Experimental autoimmune encephalomyelitis (EAE)

EAE is a models of autoimmune CNS damage. EAE is induced by the immunization of experimental animals with CNS components in the presence of an adjuvant (Baxter, 2007), to promote pathogenic autoreactive T cell responses. Studies performed on MyD88^{-/-} mice demonstrate resistance to EAE. These mice displayed also a decrease in IL 6 and IL 23 production by DCs and decreased IFNγ and IL 17 production by T cells (Prinz et al., 2006; Marta et al., 2008). Suggesting that innate immune responses are initiated through TLR or IL 1R signaling are required for the induction of experimental autoimmunity. In addition mice with lymphoid cell-specific TLR2 deficiency are less vulnerable to EAE and have lower Th17 responses compared with wild-type controls (Reynolds et al., 2010).

These suggests that the TLR pathway is very important in autoimmune disease pathogenesis. Thus, targeting TLRs and/or their signalling pathways may provide an effective therapeutic approach for certain autoimmune diseases (Hosseini *et al.*, 2015).

1.2 Dendritic cells

In 1973, the immunologist Steinman and cell biologist Cohn, described a new population of heterogeneous cells: Dendritic Cells. DCs were observed in mouse peripheral lymphoid organs and are characterized by "*pseudopods of varying length, width, form, and number, resulting in a variety of cell shapes ranging from bipolar elongate cells to elaborate, stellate or dendritic ones*" (Steinman and Cohn, 1973). This population of hematopoietic cells, with macrophages and B cells, belong to the family of antigen presenting cells (APC).

DCs are the cellular player to translate innate information into adaptive immunity, they express a big repertoire of PRRs and after signals from these receptors initiate a complex phenotypic and functional transformation called "DC maturation". As APCs, DCs have evolved different ability to efficiently take up particles and pathogens. Among these they develop different type of endocytosis for antigen accumulation:

- Phagocytosis, for ingestion of big particles or cell >1 μm. These big structures bind specific receptors on the membrane and start an actin assembly that drives pseudopod extension for particle engulfment.

- Macropinocytosis is also actin-dependent. Is important for uptake of large quantities of extracellular fluid and fluid-dissolved antigens.

- Clathrin-coated vesicles and calveolin containing invagination for soluble receptors-ligand complex.(Trombetta and Mellman, 2004)

After internalization, by any of these mechanisms, antigens are carried in endosomal and lysosomal vescicles, where they are processed by proteases into small immunogenic epitopes and associated with major histocompatibility complex molecules class I (MHC-I), newly synthesized or recycled from plasma membrane. Once associated with MHC-I are transported to the DC plasma membrane and activate naïve T cells.

Endogenous protein from cytosol or organelles are imported into lysosome by autophagy for loading onto MHC-II (Trombetta and Mellman, 2004).

1.2.1 Dendritic cells classification

DCs are a heterogeneous population of hematopoietic cells. Defined by anatomical location, cell surface marker expression and functional responses. Based on these characteristic DCs are divided in four main categories: conventional DCs (cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs and Langherans cells (Shortman and Naik, 2007).

cDCs are subdivided in two categories based on the path that they follow to reach the lymphoid organs: migratory DCs and lymphoid-tissue resident DCs. CDC are characterized by short half-life (3-5 days), continuous replacement from bone marrow precursors, expression of many PRRs and endocytic receptors, high phagocytic ability and specialized antigen-processing and presenting features. CDC are located in lymphoid, interface, and connective tissues (Waskow *et al.*, 2008). Differentiation from immature progenitors in cDC occurs when small amount progenitors transit from bone marrow to spleen and lymph nodes via the blood, after various rounds of cell division they differentiate into cDCs. Homeostasis in peripheral lymphoid organ is dependent on cell division, cell death and the rate of DC progenitor input from blood. Upon differentiated cDCs encounter antigens and capture it in peripheral tissues, they migrate via afferent lymphatic vessels to the T cell zones of secondary lymphoid organs to initiate adaptive immune responses.

cDCs subtypes. The common definition of DCs is on one hand don't express hematopoietic lineage markers (Lin$^-$) and on the other hand express high amounts of integrin CD11c (CD11c$^+$) and MHC class II (MHC-II^{high}). The problem is that this definition in misleading and poor. CD11c$^+$ is expressed by some T and B cells, macrophage population, and NK cells. MHC-II^{high} is shared by B cells and activated macrophages. In the last years some progress has been made in understanding the origin and the transcriptional pathway that controls DC differentiation. It has been shown that there are two DC population defined by the transcription factors Batf and IRF8 sharing a common origin: lymphoid tissue CD8α$^+$CD11b^{low} DCs and non-lymphoid tissue CD103$^+$CD11b^{low} (Hildner *et al.*, 2008; Edelson *et al.*, 2010) and this differentiation is inhibited by a DNA protein Id2. Important feature is that differentiation and expansion is promoted by the mammalian target of rapamycin (mTOR) and they lack macrophage markers expression. The most important characteristic shared by CD103$^+$CD11b^{low} and CD8α$^+$CD11b^{low} DCs is their functional

pattern. These cells are potent producers of interleukine-12 (IL-12), a fundamental cytokine to activate $CD8^+$ T cell. Another important function is the ability to cross-present antigens to $CD8^+$ T cells and inducing their differentiation to $CD8^+$ effectors T cells (del Rio *et al.*, 2007; Heath and Carbone, 2009).

pDCs are in general peculiar for their strong capacity to secrete rapidly large amounts of type-I interferons, they circulate in blood and lymphoid tissues (Colonna, Krug and Cella, 2002). PDCs derive from bone marrow progenitors, require Flt3L for differentiation. After activation, pDCs change radically their appearance, passing from a spherical shape to a dendritic morphology, an observation that formed the basis for their original designation as DC precursors. PDCs, differentially from cDCs, constitutively express IRF-7 that allows the fast secretion of huge amounts of IFN-α after TLR7 and TLR9 stimulation. In recent years, pDCs have become object of intensive studies that centred them as pivotal in controlling immunity and diseases, both because their IFN-α production capacity and antigen presenting properties.

Monocyte-derived DCs (mDCs) are cells that originate from blood monocytes in response to inflammation or infection, short lived and differentiate into 'Tip-DCs' (tumor-necrosis factor– and inducible nitric oxide synthase–producing inflammatory DCs). Like cDCs, Tip-DCs – up-regulate their expression of CD11c and MHC class II and efficiently stimulate naive T cells and are thought to represent the main inflammatory cell type during infection (Serbina *et al.*, 2003).

Langerhans cells (LC). Only these DCs are present in the epidermis. Important as a barrier to block early access to skin pathogens as well as to epidermal self-antigens. LCs transport epidermal self-antigens to regional lymph nodes and they present to naive T cells to initiate adaptive cutaneous immune responses. Characteristic marker of LCs is Langerin (CD207) (Valladeau *et al.*, 2000).

1.2.2 TLRs in DCs and their role in immunity

TLR triggering has pleiotropic effects on DCs. TLRs signaling promote survival, expression of chemokine receptors, chemokine secretion, cytoskeletal shape changes, migration and endocytic remodeling (Akira, 2003). Furthermore, TLR activation and signaling regulates three categories of signals that DCs can deliver to support T cell clonal expansion and their differentiation into effectors, (conveniently referred to as signals 1, 2 and 3): antigen presentation, co-stimulation and cytokines secretion (Kaliński *et al.*, 1999). Different studies of DCs subsets have revealed that TLRs have distinct expression patterns. PDCs preferentially express TLR7 and TLR9, whereas mDCs or cDC express TLR1, TLR2, TLR3, TLR5, TLR6 and TLR8 and TLR9 (Iwasaki and Medzhitov, 2004).

Whatever TLR is expressed on different subsets of DCs, the activation mechanism and maturation are similar. Upon TLR activation, DCs migrate from peripheral tissues, to the draining lymph nodes to present to naive T lymphocytes the processed peptides in the context of MHC molecules. This migration is mediated by TLR mediated up regulation of receptors for lymphoid chemokines, such as CCR7, and down regulation of inflammatory chemokine receptors like CCR6 (Dieu *et al.*, 1998; Sallusto *et al.*, 1998). During migration, DCs also undergo a maturation program and once inside the lymph nodes, seek out antigen specific T cells and induce their activation and differentiation. The main pathway by which different DCs become mature to provide the second signal to naive T cells occurs by recognition of PAMP via the TLRs (Kumar, Kawai and Akira, 2009). Each DCs population will respond only to the pathogens for which they have appropriate TLRs. In addition, stimulation through TLRs can result in a distinct outcome, even when triggered by the same ligand. For example the same synthetic TLR7 agonist can induce secretion of IFN-α and IL-12 of human pDCs and mDCs respectively (Ito *et al.*, 2002). Similarly, stimulation with TLR9 synthetic oligonucleotides containing CpG motifs induces mDCs to secrete IL-12 and pDCs to secrete IFN-α (Iwasaki and Medzhitov, 2004). Although these differences in cytokine secretion by different DCs subsets, signals throughout TLRs generally result in the maturation and activation of all DCs, as measured by improved expression of the costimulatory molecules CD40, CD80 and CD86. Induction of the costimulatory molecules on the DC surface is a particularly important step in the initiation of adaptive immunity.

1.2.4 Type-I interferon diseases

After activation and maturation DCs are able to produce IFN. The abundant and fast production of type I IFN by DCs is due to selective expression of TLRs and by a peculiar compartmentalization of the signaling pathway. As mentioned before, the binding of nucleic acid to endosomal TLR9 and TLR7 recruits MyD88 that after a phosphorilation cascade ultimately with transcription of type- I IFN genes. In 2005 Honda and colleague demonstrated the importance of a compartmentalization spatiotemporal regulation and trafficking through the endocytic pathway downstream of TLR9 engagement (C. Honda et al, 2005). They demonstrated that a prolonged preservation, of multimeric nucleic acid complexes, in early endosomes provided a platform for extended activation and transcription of the type-I IFN genes, while the rapid translocation of these monomeric nucleic acid structures to late endosomes and lysosomes failed to induce type-I IFNs production and was associated to NF-kB activation and consequent pDCs maturation. Even though pDCs activation and type-I IFN production are critical for a capable antiviral response, some studies indicate that a constant secretion of type-I IFN without infection may result in the establishment of a chronic inflammatory environment. This environment may predispose to autoimmune manifestations and human diseases such as Sjogren's syndrome and systemic lupus erythematosus (SLE), previously described (chapter 1.1.5)

Sjogren's syndrome (SS)

Sjogren's syndrome is a chronic autoimmune disease characterized by periductal lymphocytic infiltration of the exocrine glands, such as lacrimal and salivary glands, resulting in lack of their function and consequent dry eyes and mouth. Manifestations of this syndrome are rather common: cutaneous vasculitis, glomerulonephritis and peripheral neuropathy. Some studies have discovered an activation of the type-I IFN pathway as major factor for the pathogenesis of Sjogren's syndrome (Zheng *et al.*, 2009). Another studies found that polymorphisms in genes involved in the IFNα pathway, such as STAT4 and IRF5 are associated with disease susceptibility (Nordmark *et al.*, 2009). Until now initial triggers of the innate immune response in Sjogren's syndrome remain unclear, preliminary evidence supports the role of abnormaly expressed retro elements as potential triggers of type I IFN activation in the syndrome, possibly through TLR-dependent pathways (Mavragani *et al.*, 2007).

1.3 The cytoskeleton

The cytoskeleton is a highly interconnected network that regulates cell shape, division and most cellular functions. It is also crucial for immune purpose, including intracellular interactions, trafficking, endocytosis, signal transduction, cytockinesis and cell morphology. The cytoskeleton in eukaryotic cells is composed by three structures: microtubules made of tubulin, microfilaments made of actin and intermediate filaments (Wickstead and Gull, 2011). Microtubules are involved in the formation of the mitotic spindle, flagella and cilia and in the intracellular transport of organelles such as mitochondria. Microtubules are cylindrical structures formed by polymers of α- and β-tubulin organized by centrosomes, microtubule organizer center (MTOC) and basal bodies. Intermediate filaments are the more stable components and serve to maintain cellular shape, includes about 70 different proteins of which vimentin is the most common.

Actin filaments is important to maintain assembly membrane protrusions, cell morphology, cytokinesis, endocytosis, and forming cell-to-cell or cell-to matrix junctions, better describe in the next session of this manuscript.

1.3.1 The actin cytoskeleton

In eukaryotic cells actin is one of the most abundant protein and its concentration can reach up to 300μM (Blanchoin *et al.*, 2014). Actin is present in cytosol as monomers, or globular actin (G-actin), these monomers are assembled in protofilaments. Actin microfilaments or filamentous actin (F-actin) are composed by two protofilaments associated in a right-handed helix and monomers oriented in the same direction that provides to actin filaments a polar phenotype. Actin polymerization might occur at both extremities. F-actin exhibit a fast growing end the barbed end and the opposite slow-growing end called pointed end, corresponding to degrading part of filaments (Pollard and Cooper, 2009). Dynamic of actin filaments depends on continuously elongation and depolymerization. Elongation (nucleation) results from the addition at the berbed end of ATP-G actin that is quickly hydrolyzed in ADP. Filament depolymerization on the pointed end occurs when ADP-G actin

is removed. Capping proteins as well as severing proteins that induce depolymerization (ADF/Cofilin) are important to regulate the length of actin filaments.

It has been shown that spontaneous assembly rarely occurs within cells. Porfilin is a protein that binds G-actin to prevent spontaneous polymerization of F-actin. On the other hand, the activity of F-actin assembly is under control of proteins referred to as actin nucleators. The most studied actin nucleators factors in eukaryotic cells are protein from the Formin family and the Actin-Related Protein-2/3 (Arp2/3) (Carlier *et al.*, 2015). These two types of nucleator factors generate two different types of actin structures. Formins is important to stabilize actin oligomers that are already formed therefore promotes their elongation in bundles. Arp2/3 binds to the side of an existing actin filaments and promotes the polymerization of a new filament, branched networks (*Figure 1.3*).
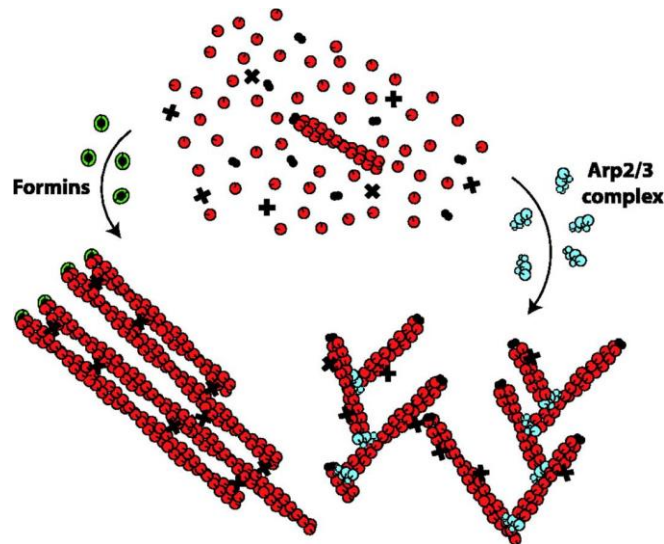

Figure 1.3. Formin and Arp2/3 mediated actin nucleation. Actin network geometry depends on how long the barbed end is free during filament nucleation. Starting with the same building blocks, the formins and Arp2/3 complex will create very different networks because of their opposite treatment of barbed ends. Arp2/3 complex (blue) nucleates filaments with free barbed ends from the sides of pre-existing filaments. Because these filaments are quickly blocked by capping proteins (black), the resulting network is composed of short filaments arranged in a brush-like structure. Such networks are seen, for example, at the leading edges of crawling cells. By contrast, formins (green) protect barbed ends from capping as they grow to create long filaments that can be arranged into bundles by cross-linkers (x).

From Bindschaldler and McGrath, 2004

1.3.2 Arp2/3 complex

In 1994 Machesky and colleague identified seven polypeptides that interacts with Profilin (actin-binding protein) and all together composed one complex (Machesky *et al.*, 1994). Two of these proteins where named actin-related protein 2 and 3 (Arp2 and Arp3), respectively since they are structurally similar to G-actin. This is the origin of the name of Arp2/3 complex. The other five subunits, Arp2/3 complex component 1-5 (ArpC1-5), act as scaffold proteins. These proteins provide to the complex the correct organization to support actin nucleation and bind the site of an existing actin filament. Arp2 and Arp3 start the addition of first actin dimer of a new filament with a branch angle of ~70°. After nucleation, actin filaments continue to spontaneously extend until terminated by capping proteins. Capping activity is modulated by anti-capping proteins, such as ENA and vasodilator-stimulated phosphoprotein (VASP), which, by protecting the growing barbed end from abundantly present capping proteins, promote continued filament extension (Chesarone and Goode, 2009).

Arp2/3 complex by itself is inefficient at activati nucleation, for its activation require other protein called nucleation promoting factors (NPFs) (Firat-Karalar and Welch, 2011). NPFs are divided into two different classes depending on their mode of y-branching reaction and on the mechanism by which they activate the complex Arp2/3: class I and class II.

Class I NPFs. The first NPF identified was ActA, a protein found on the surface of *Listeria monocytogenes*, required for actin-based bacterial motility in the host cytoplasm. Characterization of this protein was quickly followed by the identification of eukaryotic class I NPFs: Wiskott Aldrich syndrome protein (WASp), neural WASp (N-WASp), suppressor of cyclic AMP repressor (SCAR; also called WASp-family verprolin-homologous protein (WAVE1-3)) (Goley and Welch, 2006). WASp is restricted to hematopoietic cells, is important for podosome formation and internalization step of endocytosis. WAVE proteins are necessary for the formation of membrane ruffles and lamellipodia. In the last years, exiting developments include the discovery of new type I NPFs: WHAAM (WASp homolog associated with actin, membranes, and microtubules) required to preserve Golgi morphology and help transport along biosynthetic pathway, WASH (WASp and Scar homolog) important for endosome fission and JMY (junction

mediating and regulatory protein) that regulates transcription and cell migration (Derivery *et al.*, 2009; Rottner, Hänisch and Campellone, 2010). Class I NPFs will be better describe in the next session.

Class II NPFs include meatazoan cortactin and *S. cerevisiae* actin binding protein-1 (Abp1) and Pan. The mechanism of Arp2/3 activation by this class is not entirely clear and is less potent activators than class I. Class II is not detailed in this manuscript.

1.3.3 Nucleation-promoting factors class I

NPFs class I is a group with evolutionary conserved regions. Each of these possess C-terminal domain (VCA) composed by verpolin homology sequence (V), central connecting sequence (C) and acid region (A). Verpolin sequence binds actin monomers and acid one binds Arp2/3 complex inducing change in conformation. N-WASp and WHAMM have two verpolin homology sequence, while JMY possess three of this sequence. At the N-terminal region there are several other regulatory domain with differences among all NPFs (Burianek and Soderling, 2013) (*Figure 1.4*).

- WASp and N-WASp are approximately 500 amino acid cytosolic proteins and are structurally equal; there is WASp homology domain (WH1), lysine riche basic region (B), GTPase binding domain (GBD) and a proline-rich domain (PRD).

-WAVE possess WAVE homology domain (WHD) instead of GBD and there is no basal actin nucleation activity.

- WASH is very different at N-terminal domain. Has WASH homology domain (WAHD1), tubulin binding region (TBR), and PRD.

- WHAMM and JMY maintain PRD but they have coile coil (CC) region and WHAMM present a WHAMM interaction membrane domain (WMD) whereas JMY N-terminus (N) region.

Figure 1.4. Domain structures NPFs family proteins. All of the WASP family members exhibit a proline rich region and a VCA tail in the C-terminus, but contain unique N- termini. WH1: WASP Homology domain, B: Basic domain, GBD: GTPase binding domain, PRD: Proline rich domain, V: Verprolin homology, C: Connecting sequence, A: Acidic sequence, WHD: WAVE homology domain, WAHD1: WASH Homology domain, TBR: Tubulin binding region, WMD: WHAMM membrane-interacting domain, N: N-terminal region.

From Burianek and Soderling, 2013.

Although in the last years there is an increase in studies to understand NPFs activity, the specific mechanism enabling Arp2/3 activation by NPFs class I is still lacking. However, has emerged as a common model and is now well-accepted.

Upon Arp2/3 binding by VCA domain induces a conformational change in the Arp2/3 complex that brings Arp2 and Arp3 closer to each other mimicking a dimer of G-actin, which allow actin-nucleating activity and elongation of the new filament at the barbed end. (Goley and Welch, 2006; Chesarone and Goode, 2009). Activation of the Arp2/3 complex mediated by NPFs is a highly regulated mechanism controlled by the Rho family of small GTP-binding proteins. These GTP-binding proteins includes Rho, Rac and Cdc42.

1.3.4 NPFs in membrane trafficking

The different classes of nucleation-promoting factors discussed above play important roles in a variety of cellular processes such as membrane trafficking, leading edge protrusion and cell division. Here I will focus on recent advances on membrane trafficking.

Actin polymerization is important in several membrane trafficking events including endocytic internalization, endocytic transport, and ER to Golgi transport. One function common to each of these processes is the dynamic shaping and remodeling of membranes. The actin nucleators that contribute to these include the formins and Arp2/3 complex with its NPF (Firat-Karalar and Welch, 2011). (*Figure 1.5*).

Actin polymerization and its nucleation is important to generate initial force that drives the shaping of membrane invaginations and the development of PI(4,5)P2 lipid phase segregation. This lipid phase separation generates an interfacial force that constricts the membrane invagination and helps vesicle scission. It was recently shown that actin polymerization by the Arp2/3 complex and activated **N-WASp**, without other factors, drive vesicle scission from tubulated membrane intermediates in a reconstituted system in vitro (Romer *et al.*, 2010). This study support the emerging view that actin induces membrane deformation and scission via a capacity to generate force and/or promote lipid phase segregation (Firat-Karalar and Welch, 2011).

The role of actin in the later stages of endocytic trafficking is poorly understood and the molecular players are just now being identified. **WASH** is a nucleating protein relevant for the early-to-late endosome transport. WASH localizes to patches on early and recycling endosomes, where it promotes actin nucleation activating Arp2/3 complex (Derivery *et al.*, 2009; Duleh and Welch, 2010). WASH is found primarily on early and slow recycling endosomes and little amount of this protein on fast-recycling and late endosome (Rottner, Hänisch and Campellone, 2010). WASH is also involved in modulating endosome shape and it is hypothesized to function in receptor recycling trafficking, although how the activity of the complex is regulated has not yet been determined (Derivery *et al.*, 2009; Firat-Karalar and Welch, 2011; Mesaki *et al.*, 2011). In addition, actin nucleation has also been implicated in ER-to-Golgi transport. **WHAMM** localizes in the cis-Golgi and is important to maintain Golgi shape and facilitating ER-to-Golgi transport (Campellone *et al.*, 2008). Moreover, WHAMM localizes to tubulo-vesicular membrane transport intermediates promoting membrane tubulation and tubule elongation(Firat-Karalar and Welch, 2011). Although WHAMM is the best-characterized way of nucleating actin during ER- to-Golgi transport, the formin INF2 was shown localized to the ER and is implicated in actin assembly in cells (Firat-Karalar and Welch, 2011).
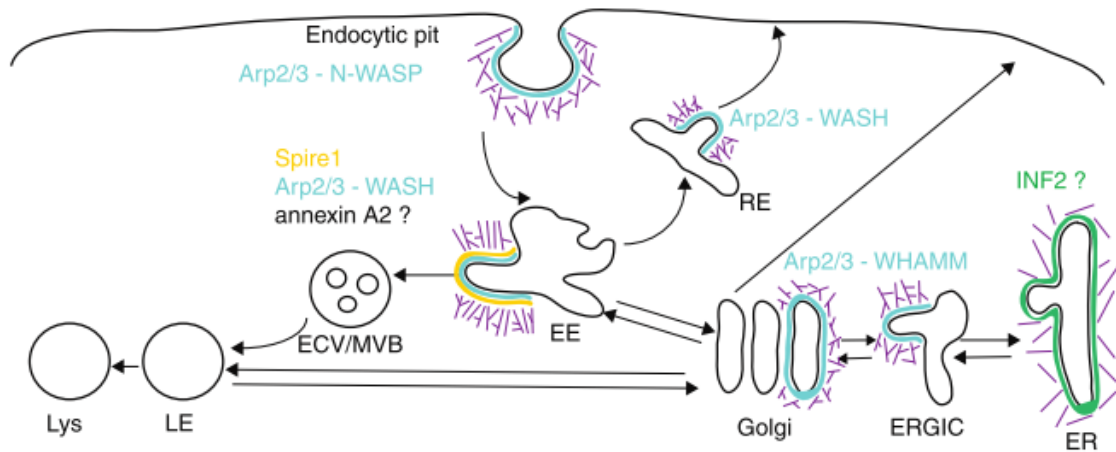
Figure 1.5. Models of the cellular localization and function of actin nucleators. A depiction of the role of actin nucleators in membrane-trafficking events including endocytic internalization as well as various stages of endocytic and ER-to-Golgi trafficking. Abbreviations: EE, early endosomes; ECV/ MVB, endosomal carrier vesicles/multivesicular bodies; LE, late endosomes; RE, recycling endosomes; Lys, lysosome; ER, Endoplasmic reticulum; ERGIC, endoplasmic reticulum-Golgi intermediate compartment.

Adapted from Firat-Laralarand Welch, 2011.

Future studies will help to address the important role of actin nucleation in membrane trafficking by exploring more precisely the stages of transport, the cargo that requires actin and what biophysical role actin nucleation plays in reshaping and remodeling membranes.

1.3.5 WASp expression and regulation

In the last year some studies have revealed important pathological consequences linked with alteration in actin regulatory genes. Wiskott-Aldrich Syndrome (WAS) is a rare X-linked recessive primary immunodeficiency and remarkable demonstration of the importance of a well-regulated actin polymerization to support immune cell function. WAS derives from mutations in Wiskott-Aldrich Syndrome protein (WASp) and is associated with a variety hematopoietic cell defects. WASp is a protein with actin-polymerizing activity, restricted on hematopoietic cells. For this reason, it is a suitable model to investigate the role of cytoskeleton alteration in immune cells and pathology.

Structural studies demonstrate that WASp is present, in the cytosol, in an auto-inhibited conformation. This inhibition is created by intermolecular ad intermolecular interaction that alters its affinity for Arp2/3 complex (Kim *et al.*, 2000). Binding between VCA domain and

hydrophobic pocket in the GBD of WASp prevents full length WASp from binding with and activating the Arp2/3 complex. This interaction is reinforced by WASp-interacting protein (WIP) binding with WH1 domain, stabilizing WASP molecule and also suppressing their capacity for activating Arp2/3 activity. WASp activation occurs when GTP-Rho family GTPase cell division cycle 42 (Cdc42) bind GBD, this interaction leads to the release of WIP from WH1 and VCA from GBD, allowing binding by the Arp2/3 complex and actin nucleation. Another important step to modulate WASp is phosphorylation of at a conserved tyrosine residue 291 (Tyr291) (Cory *et al.*, 2002; Thrasher and Burns, 2010). In the auto inhibited structure, Tyr291 is inaccessible to tyrosine kinases. After CDC42 binding, WASp opened allowing phosphorylation by SH2-containing tyrosine kinases, such as Src. Dephosphorylation of this site by the tyrosine phosphatase PTP-PEST abrogates the inducible coupling of WASp to actin polymerization (Badour *et al.*, 2003) (*Figure 1.6*).
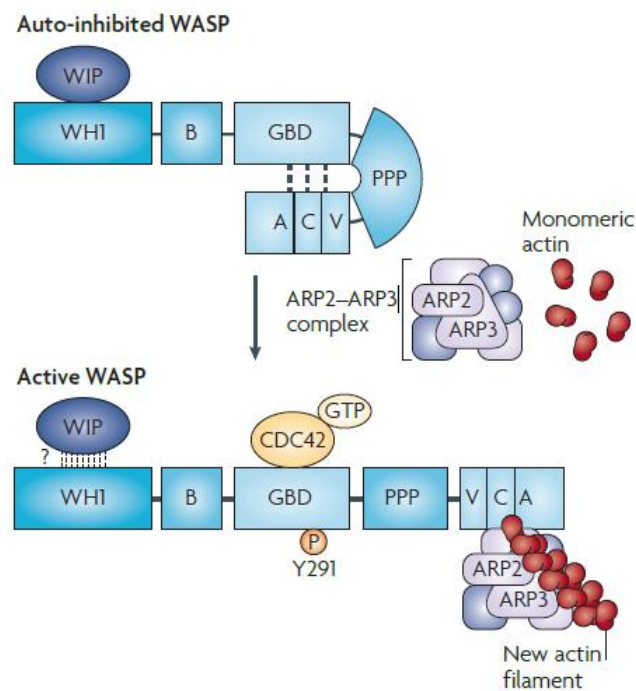

Figure 1.6. Domain structure of Wiskott–Aldrich syndrome protein. In its normal state, WASP has an auto-inhibited conformation in which an intermolecular interaction between the VCA domain and GBD is thought to prevent binding of the Arp2/3 complex and monomeric actin to the carboxyl terminus. The association WIP with the WH1 domain is thought to stabilize the auto inhibited configuration, although it is not clear whether WIP remains bound following WASP activation. The Rho family GTPase cell division cycle 42 (CDC42) is the main WASP activator, by binding to the GBD. Phosphorylation of WASP tyrosine residue291(Y291) might either activate WASP alone or stabilize an active conformation initiated by other factors.

From Thrasher and Burns, 2010.

The density and complexity of such regulatory mechanisms and the increasing number of protagonists involved in these processes highlight how crucial it is for the cell to tightly control in time and space the assembly of actin filaments.

Additional work is still needed to better understand the accurate mechanisms allowing the fine regulation of WASP. Activation at different subcellular localizations represents another level of regulation that allows building particular actin networks that serve specific cellular functions.

1.3.6 Immune cellular function dependent on actin cytoskeletal dynamics

Actin cytoskeleton is very important for the cells since participates in a lot of different cellular processes as it provide the basic framework for a large range of subcellular structures and for appropriate immune functions. These functions include cell-cell interactions, membrane motility, cell migration, and endocytosis.

<u>Cell migration and actin cytoskeletal structures in membranes.</u>

Cell migration is one of the most studied actin-dependent processes. Amoeboid cell migration depends on cycles of actin polymerization at the front edge and contractility at the opposite part of the cell. Immune cell migration is important for mediating tissue specific cell trafficking and the outcome of immune responses. Immunocytes utilize various actin structures in membrane to promote forward motion during migration and tissue invasion, such as filopodia, podosomes and invadopodium (de Noronha *et al.*, 2005; Bouma, Burns and Thrasher, 2007; Blundell *et al.*, 2008; Thiam *et al.*, 2016)

Filipodia are thin actin cytoskeletal projections made up of both linear and branched-chained F-actin. Different actin cytoskeletal proteins regulators have been implicated in this process: Cdc42 and N-WASp. It was shown that N-WASp localizes to certain types of filopodium, however, this protein is not so essential in the assembly process because some filopodia still form in absence of N-WASp (Snapper *et al.*, 2001).

Podosomes and **invadopodium** are protease-rich protrusion utilized by macrophages, monocytes, and metastatic cancer cells during tissue invasion, this structure facilitates degradation of the extracellular matrix allowing cells to advance. N-WASp is required for

invadopodium and podosome formation, in a negative mutant of N-WASp in Src-transformed cells was found to inhibit of invadopodium structure and degradation of the extracellular matrix. Furthermore the binding between cortactin and N-WASp regulate this process by the recruitment of N-WASp to the site where Arp2/3 is localized, resulting in a strong activation and podosome formation (Mizutani *et al.*, 2002). Tsuboi and colleague showed in macrophages that a complex of WASp and interacting protein WIP is essential for podosome formation (Tsuboi, 2007). In another study Olivier and colleagues shown that a partial reduction of WASP level was sufficient to inhibit development of podosomes in dendritic cells (Olivier *et al.*, 2006). Some years later it has been shown that WASp is important to promote correct podosome array organization by DCs (Dehring *et al.*, 2011).

Cell-cell interactions

Actin cytoskeleton and its regulation is also critical for intracellular interactions, such as immune synapse between DCs and T cells. At the immune synapse there is a dense actin network which is important to assure a tight cell adhesion. WASp is crucial to control ex-novo actin polymerization necessary to stabilize synapse formation and signaling. Alteration in WASp expression is associated with altered assembly of actin filamentous and impaired recruitment of several immunological synapse proteins in response to TCR stimulation (Dupré *et al.*, 2002; Sasahara *et al.*, 2002).

Our group has also discovered a role long-lasting stable interaction and proper transmission of activating signals for WASP on the DCs side of the immunological synapse (Pulecio *et al.*, 2010).

Internalization processes: Endocytosis

Internalization of extracellular particles or fluid represents a crucial path of entry within cells and particularly applies for cells of the immune system. Among these processes, phagocytosis endocytosis and macropinocytosis are key entry routes. While macropinocytosis represents a non-specific entry route, endocytosis and phagocytosis are mediated by the engagement of specific receptors at the cell surface.

Endocytosis is a cell-surface membrane trafficking event that brings soluble molecules, receptors or membrane components to the endocytic pathway. Exist two endocytosis mechanisms: clathrin-dependent and clathrin-independent endocytosis (such as

macropinocytosis, caveolar endocytosis and phagocytosis). In the first case vesicles internalized are coated with clathrin and the heterotetramer Assembly Protein 2 (AP2), and accessory proteins such as epsin. Epsin is important to link receptors to coat components and contribute to the bending of the lipid bilayer (Ford *et al.*, 2002). Another important protein in both clathrin-dependent and clathrin-independent endocytosis is Dynamin, involved in vesicle fission from donor membrane. After fission from plasma membrane vesicle coated loose clathrin and recycled for another transport, instead uncoated vesicle fuse together to form new endosomes, or fuse with pre-existing early endosomes (EE) (Maxfield and McGraw, 2004).

EE are very important for cells, they are entry portals, sorting stations and signaling platforms and they direct molecules into the appropriate pathway: degradative and recycling. Molecules for degradation are sorted into specific vescicles that start maturation process with acidification and acidification and formation of compartments named multivescicular bodies (MVBs) (Futter *et al.*, 1996). These MVBs maturate in late endosome (LE) and fuse with lysosomes where proteases begin cargo degradation and signal switch off.

Molecules for recycling are directly transported to the plasma membrane by recycling endosomes (RE) (Maxfield and McGraw, 2004). In all this process of endocytosis, actin polymerization seems to have important roles in vescicle fission and in subsequent vescicle trafficking inside the cell. Actin was shown to provide a motile force that supports fission activity (Roux *et al.*, 2006), and promote endosome morphology and movement (Ohashi *et al.*, 2011).

The precise role of actin in endosome dynamics and biogenesis is not clear, altought several possible scenarios can be evoked. They include remodeling of the actin network by endocytic vesicles along their trajectory, regulated endosome anchoring onto the actin network at the cell periphery, endosome motility along existing actin filaments, and possibly of de novo F-actin formation. Otherwise, actin could be active in membrane remodeling during endosome biogenesis, but little is known about the mechanisms that control physical interactions between endosomal membranes and actin. Morel and colleague shown the role of actin in transport from early to late endosome. They find that patches of F-actin, regulated by actin nucleation factors and Arp2/3, are nucleated near early endosomes and are required for transport beyond early endosomes (Morel, Parton and Gruenberg, 2009).

1.4 Wiskott Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS, OMIM 301000) is a rare, complex and severe X-linked primary immunodefiency which is caused by mutations in hematopoietic specific regulator of actin nucleation, WASp. Mutation in WAS gene are different and lead to variations in the syndrome. Premature termination or deletion completely abrogate WASp and lead to severe disease WAS. Reduced protein expression derived from missense mutations leads to phenotypically milder X-linked thrombocytopenia (XLT). Eventually mutation in GBD domain disrupts the auto inhibited conformation generating a constitutively active protein and results in X-linked neutropenia (XLN) (Devriendt *et al.*, 2001; Blundell *et al.*, 2010; Moulding *et al.*, 2013). WAS patients develop a high incidence of eczema (about 80%), thrombocytopenia, increased risk of infection and malignancy and predisposition to immunodeficiency. In patients there is a high incidence of immunodeficiency (40-72%) and the most common diseases are vasculitis, autoimmune hemolytic anemia, renal disease, arthritis, neutropenia and inflammatory bowel disease (Ochs and Thrasher, 2006; Bosticardo *et al.*, 2009; Catucci *et al.*, 2012) In addition, gain-of-function mutations in the WAS gene, that lead to a constitutively active protein, are found to cause the X-linked neutropenia, characterized by low neutrophil number and predisposition to myelodysplasia in the absence of thrombocytopenia and T-cell immunodeficiency (Notarangelo *et al.*, 2002; Ancliff *et al.*, 2006)

All these clinical manifestations highlight the complexity in WAS and important role of WASp in various cellular mechanisms. WASp is expressed by all hematopoietic cells and precursors cells, however importance of WASp change at different stages of hematopoietic cell development affecting survival, cell migration and differentiation. A lack of WASp results in cytoskeletal defects that compromise different aspects of normal cellular activity, this cause defect in homing, migration, abortive cup formation in phagocytic cells, proliferation, phagocytosis, immune synapse formation, adhesion and is also involved in polarization and secretion of cytokines. To better study mechanisms and disease WASp deficient mice (WKO mice) were generated providing helpful model. WKO mice exhibit similar defects in hematopoietic cells, however clinical manifestations differ somewhat. WKO mice present primarily immunodeficiency, thrombocytopenia with only little inflammatory

bowel disease (Zhang *et al.*, 1998) and a high incidence of antinuclear antibodies with high levels of anti-DNA (Humblet-baron *et al.*, 2007).

1.4.1 Cellular defects in WAS

WAS arise from different type of mutation in the gene that codes for WASp. Initial studies on T cell activation, regulation and function have contribute to the understanding of molecular basis of disease in WAS patients. Not only T cell contribute to the pathogenesis but is also evident that there is a general impairment of hematopoietic cell functions.

T cell

Defects in T cells play a crucial role in WAS-associated immunodeficiency. Lack of WASp in mature T cells is associated to fewer microvilli and marked migration deficiency with poor adhesion and reduced IL-2 production. Actin cytoskeleton remodeling by WASp play a key role in T-cell activation, in the engagement of TCR, co-stimulatory molecules CD28 and CD2 (Gallego *et al.*, 1997; Badour *et al.*, 2003, 2007; Morales-Tirado *et al.*, 2010). In absence of protein there is a failure of proliferation in response to immune activation following TCR ligation in the context of immune synapse (IS). IS in WASp knock-out (WKO) condition can be formed only after strong TCR stimulation (Cannon and Burkhardt, 2004). WASp null cells (WKO cells) fail to polarize normally cytokines toward a target in the cells and show a variable block in their secretion (Morales-Tirado *et al.*, 2004). Different studies described functional alterations of regulatory T cells (Treg), the key lymphocyte subset to maintain peripheral tolerance. WAS defective Tregs in humans and mouse models were shown to have altered homeostasis and reduced suppressive ability. *In vivo*, WKO Tregs failed to protect in a T cell transfer model of chronic colits (Humblet-baron *et al.*, 2007). Recently, the colitogenic proprieties of WKO T cells were shown to depend on aberrant cross-talk with innate immune cells, suggesting that Treg dysfunctions are not completely T cell intrinsic (Nguyen *et al.*, 2013).

B cells

In the last years several studies have investigated the role of WASp in B cells. B-cell anomalies are mainly focused on on the defective cytoskeletal-dependent processes of WKO

cells leading to decreased adhesion, migratory homing and ability to assemble long protrusions (Westerberg *et al.*, 2001, 2005). Accordingly with decreased motility, of WKO B cells, has been evaluated the main cause of selective depletion of circulating B-cell in peripheral blood and secondary lymphoid organs in both WAS patients and murine WKO model (Westerberg *et al.*, 2001; Park *et al.*, 2004; Thrasher and Burns, 2010). In WAS patient there is an evident reduction in the expression of CD21/CD35 receptors responsible of ruin capability of antigen capture and presentation. Furthermore there is a marked reduction in amount of CD27+, indicating a defective cell differentiation (Park *et al.*, 2005). Some different studies unveiled an important role of WASP in peripheral homeostasis of mature B-cell subsets. It seems that WASp is dispensable for early B-cell development. When WASp is absent there is an impairment for completion of B-cell maturation (Park *et al.*, 2005; Thrasher and Burns, 2010). In addition, WASp seems to be essential for early B-cell development, whereas its deficiency is harmful for completion of B-cell maturation, starting from transitional stage and affecting splenic marginal zone and peritoneal B1a cells. This phenotype appears to be triggered by a defective homeostasis and/or retention of mature B cells, rather than increased apoptosis of WKO B cells (Notarangelo *et al.*, 2008; Becker-Herman *et al.*, 2011). Moreover, indication has begun to arise demonstrating a role for B cell-intrinsic mechanisms in WAS-associated autoimmunity (Becker-Herman *et al.*, 2011).

Dendritic cells

Also, in DCs effects of mutations in WASp result in severe modifications. Lack of WASP is associated to alteration in cell adhesion, migration, T-cell priming and antigen presentation. When WASp is absent DCs presents impaired *in vivo* migration toward sites of inflammation due to failure to polarize. Many groups have showed that in DC WASp is also important for podosomes formation. Lack of WASp results in complete abrogation of podosomes in human, while in mice there is a disorganized cluster of F-actin dots resulting in a poorly ordered podosomal structures. Podosome defect resulted in a poor integrin assembly reducing adhesion to intercellular adhesion molecule-1 (ICAM-1), a ligand for β2 integrin, an important molecule for migration over the endothelium (Burns *et al.*, 2004; Calle *et al.*, 2004; Moulding *et al.*, 2013). Moreover, WKO DCs display defective chemotactic response to different chemoattractants, such as FMLP, MCP-1, since DCs failed to form lamellipodia (Zicha *et al.*, 1998; Arkwright, Abinun and Cant, 2002). Migratory response of WKO cells toward CCL21 and CCL19, highly expressed on endothelial venules and lymphatic

endothelium is severely decreased (de Noronha *et al.*, 2005). Moreover, WKO DCs are not able to spread correctly and form stable leading edge, resulting in inefficient homing into lymphonodes and defective directional migration (Bouma, Burns and Thrasher, 2007). Finally our group demonstrated that WKO DCs show defective interaction and activation of CD4+ and CD8+ T cells in lymphnodes (Pulecio *et al.*, 2010). We showed that the frequency and duration of contact between WKO DCs and T cell, *in vitro* and *in vivo*, was reduced and CD8+ T cell priming resulted inefficient. All this data are important to remark the relevance of WASp in DCs during the first phases of adaptive immune response.

1.4.2 Autoimmune manifestations in WAS

WAS-associated autoimmune complications are frequently observed (40-72%). All patients present different type of autoimmune manifestation and frequently these are multiple in the same person. Among these the most common are cutaneous vasculitis, arthritis, autoimmune hemolytic anemia, and nephropathy. Other less frequent autoimmune manifestations include dermatomyositis, recurrent angioedema, uveitis, neutropenia, and cerebral vasculitis (Dupuis-Girod *et al.*, 2003).Patients with XLT present less incidence of autoimmune diseases compare with patient with fully WAS. Autoimmunity is connected with a higher risk of a later development of tumors and an increased risk of mortality (Sullivan et al., 1994). Interestingly, a study of Monteferrante and colleague reported SLE development in one patient with WAS gene-mutated background (Monteferrante, Giani and van den Heuvel, 2009). SLE was identified because the patient fulfilled four criteria for definite SLE. Positive antinuclear antibody testing, arthritis, proteinuria, haematuria, and haemolytic anaemia with lymphopenia. The patient presented a mutation in the EVH1 domain of WAS gene, with a marked reduced expression of the specific messenger.

Until now the mechanisms of WAS-associated autoimmunity are not completely understood. It has been proposed that autoimmunity might be the result of tissue damage making by chronic inflammatory state that is established after partial pathogen clearance (Arkwright, Abinun and Cant, 2002). It was demonstrated that lack of WASp lead to defective localization and function of, CD4+ CD25+ FOXP3+ regulatory T cells (Tregs), crucial to prevent autoimmune diseases by down-regulating immune response to pathogens, allergens, and cancer cells (Humblet-baron *et al.*, 2007; Maillard *et al.*, 2007; Marangoni *et al.*, 2007).

Moreover, Rawlings group showed that mutations of the WAS gene in B cells associate with hyper-responsiveness to BCR and TLR triggering. Using a chimeric mice, B-cell restricted deletion of WASp is enough to induce production of pathogenic auto antibodies due to an intrinsic hyper-responsivness to BCR and Toll-like receptor (TLR) signals and lead to autoimmunity (Becker-Herman *et al.*, 2011). In another study, genetic deletion of WASp in B-cells confirmed that most alterations, including the production of anti-ds and anti-nuclear autoantibodies is B-cell autonomus. Our group has recently show, for the first time, a new piece of the WAS autoimmunity puzzle. We have shown that altered type-I interferon production by plasmacytoid dendritic cells (pDC) contribute to the development of autoimmunity (Prete *et al.*, 2013). Among the different DCs subsets, pDC are specialized in the secretion of high level type-I interferon upon recognition of pathogen-derived nucleic acids by endosomal TLR9. Using WKO mice it was demonstrated that WASP null pDC are intrinsically more responsive to multimeric agonist of TLR9 and constitutively secrete type-I interferon. Therefore type-I interferon production is negatively regulated by WASP-dependent actin dynamics. Lack of WASP renders pDCs highly sensitive to endogenous triggers. In the mouse model of WASp null cells, our group observed different and multiple signs of pDC activation: increased proliferation rate, elevated expression of surface maturation marker, constitutive transcription of IFN-α genes and high levels of systemic IFN-α. Moreover, we found up-regulation of several IFN-I dependent genes (IFI27, OAS1, IFITM2, IFIT1, IFI4) in peripheral blood of WAS patients.

2. Aim

A key factor controlling the induction and down-regulation of innate genes expression in DCs is the intracellular localization and trafficking of endosomal TLRs and their ligands. Although the transit of endosomal TLRs across the endocytic pathway has been elucidated to some details, the cytoskeletal forces and molecular motors involved remain poorly understood.

Previous studies in our group have shown that type-I IFN production by innate cells is increased in WAS patients and in WASp null mice. Genetic deletion of IFN-I receptor suggested that excessive IFN-I contributes to the development of autoimmunity.

The study also indicated that lack of WASp drives perturbation of an unidentified step of the endocytic trafficking leading to accumulation of intracellular ligands. However, the precise cellular mechanisms causing such perturbations and leading to increased IFN-I production downstream of TLR9 need to be clarified.

Understanding the exact molecular mechanism sustaining enhanced innate responses in WASp deficient cells is essential to understand the disease pathogenesis and to develop targeted therapies. Moreover, this disease model is a suitable tool to analyses mechanism used by innate cells to maintain tolerance to potentially dangerous endogenous triggers.

The goal of my research activity has been to define the role of WASp in the intracellular trafficking steps that ensures correct localization, activation and signal termination of TLR9 and its ligands in DCs. To this aim I have developed new cellular tools and assays to study: 1) structure and function of the endo-lysosomal system, 2) the processing and degradation of TLR9, 3) the intracellular fate and signaling induced by physiologically relevant inducers of autoimmunity; 4) the role of WASp-driven actin polymerization in controlling endo-lysosomal dynamics and TLR9 signaling.

3. Materials and Methods

Mice

The animals used in this study were six to eight weeks old. WASp-/- mice on a C57BL/6 (CD45.2) genetic background, a gift from S. Snapper (Massachusetts General Hospital, Boston, MA). Mice were bred and maintained in sterile isolators. Experiments were performed using WASP-/- homozygous female or WASp- male as WKO mice and wild type (WT) littermate as control. Mice were housed and handled according to institutional guidelines, and experimental procedures approved by the International Centre for Genetic Engineering and Biotechnology (ICGEB) board. Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European Economic Community [EEC] Council Directive 86/609; OJL 358; December 12, 1987). Protocols were approved by the Italian Ministry of Health.

Cells

Mouse bone marrow-derived dendritic cells (BM-DCs) were generated *in vitro* from BM of C57BL/6 WT or WKO mice. BM.-DCs cells were cultured at the concentration of 1.5×10^6 cells/ml in Nunc non-treated 6 well-plates (Thermo scientific) for 7 days using IMDM supplemented with 10% FBS, 50 μM 2-Mercaptoethanol (Gibco), 1% Gentamicin, complemented with 30% of supernatant of granulocyte macrophage colony-stimulating factor (GM-CSF) produced from J558 cell line. Cells were used for experiments between days 6 and 8.

Virus production and cell infection

The expression plasmid and relative packaging with envelope (Table 1) were co-transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen). Twenty-four hours after transfection, the supernatant containing virus was collected filtered and add to cells for infection. A total of 3×10^5 cells, to be infected, were dispensed in 1 ml per well in a 12-well plate and infected with virus by spinoculation at 1500g for 60 min in the presence of

Lipofectin (0.1%, Invitrogen). After spinoculation, cells were diluted by adding 1,5 ml and incubate overnight. One day after infection 1,5 ml of media was removed and replacement with 1,5 ml of fresh cell culture medium supplemented with the relative selected antibiotic for resistance.

Expression Lentivirus	Packaging	Envelope	Bacterial resistance	Cell Resistance
SEWW (Egfp-WASp)	pCMVR8.74	pMDG2	Ampicillin	Puromycin
Lenti CrisprV1	Δ8.9	VSVG		Puromycin
Expression Retrovirus	**Packaging**	**Envelope**	**Bacterial resistance**	**Cell Resistance**
MSCV herad-3HAHOXB8	######	p CL-ECO	Ampicillin	######
MSCV2.2 TLR9-HA (IRES-GFP)	######	p CL-ECO	Ampicillin	puro

Table1 Summary of lentivirus and retrovirus plasmid.
Plasmid are ordered based on expression plasmid, then the relative packaging and envelope. Retrovirus plasmid present only envelope.

SgRNA Design

SgRNAs, for WASp gene editing, were designed using the CRISPRtool: http://crspr.mit.edu. to minimize potential off-target effects. Lenti CrisprV1 plasmid, (Table 1) was digested with BsmBI restriction enzymes and three different sgRNA sequences were cloned inside. All the sequences are reported as 5'→3'

1   for   caccGGAATGTTCTGCTGAACGGC
       rev   aaacGCCGTTCAGCAGAACATTCc

2   for   caccGGATGAAGTAGGACTTCTGA
       rev   aaacTCAGAAGTCCTACTTCATCC

3   for   caccgAGCAAAAGTGTGGAAGAACG
       rev   aaacCGTTCTTCCACACTTTTGCTc

Generation of hematopoietic progenitor cell

As previously described by s Häcker group with little modification. BM cells were collected by flushing femurs of 4–8-week-old mice with 10 ml RPMI (Invitrogen), supplemented with 10% FBS (Euroclone), 50 mM 2-mercaptoethanol and 1% gentamicin, pelleted by centrifugation, resuspended in 4 ml, loaded on 3 ml Ficoll-Paque (Pharmacia) and separated by centrifugation at 1800 rpm for 30 min. The entire supernatant was collected (discarding only 500 μl including the cell pellet), diluted with 45 ml of PBS containing 1% FBS, pelleted at 2000 rpm for 10 min, followed by resuspension in 5ml PBS/ 1%FBS, pellet at 450g for 5 min and resuspension at a concentration of $\times 10^5$ cells/ml in RPMI containing 15% FBS, gentamicin, 30% GM-CSF supernatant plus 15% of Flt3L-containing supernatant, produced from an SP2/0 transfected cell line that secretes murine recombinant Flt3L. After 2 days of cell culture, cells were collected, resuspended in progenitor outgrowth medium (POM): RPMI supplemented with 10% FBS, 1% gentamicin and 1 μM β-estradiol (Sigma E-2758) and infected as describe before with MSCV herad-3HAHOXB8 (Redecke *et al.*, 2013). For one-month cells were dispensed every 3–4 days in fresh medium and transferred into new wells. After this period cells were expanded in HoxB8 Outgrowth Medium (POM supplemented with 15% Flt3 supernatant). For subsequent differentiation experiments, cells were washed twice with PBS containing FBS and resuspended in a concentration of 0.5×10^5 cells/ml in IMDM containing specific growth factors, 30% GM-CSF.

TLR agonists

DCs activation was induced stimulating cells with CpG-B (ODN 1668), CpG-B/FITC B (ODN 1826 I) and LPS were from InVivoGen, San Diego, CA. Immune Complex (IC) were from MedImmune.

Flow cytometry

The following antibodies were obtained from BioLegend: CD11c-PE/Cy7 (N418), F4/80-A488 (MCA497), MHCII-APC/A488 (AF6-120.1) CD11b-PercP-Cy5.5 (M1/70) Ly6C-PE (HK1.4). From BD Biosciences the following antibodies were purchased: CD86-PE (B7-2). MHCI-PE (AF6-88.5.5.3) was obtained from eBioscience. Viability was assessed by staining with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies). For cell staining,

FcR binding sites were blocked by using αCD16/CD32 (93, Biolegend). Samples were then stained with specific antibodies in PBS + 1% BSA and fixed with PBS + 1% PFA. For intracellular staining cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) following manufacturer's instructions, and then stained with anti-WASp-A488 (Santa Cruz Biotechnology) or anti-HA (roche). Flow data were acquired with a FACS Celesta or FACS Aria III (BD Biosciences) and analyzed with Diva software (BD Bioscience) or FlowJo software (Tree Star, Inc.).

Recycling assay (Transferrin)

Cells were starved 15' in media without FBS at 37°C, after this time cells were pulsed for 1 hour at 37°C with Transferrin 647 (50ug/ml Molecular probes) to allow cells to reach eauilibrium. Cells were moved to 4°C, washed twice in PBS cold and treated for 2'30'' with 500 µl of cold acid wash (Glycine 50mM, NaCl 150mM pH 3) reaction was stopped adding 1 ml of cold neutralization buffer (PBS, BSA 0,05%). Cells were than incubated at 37°C with non-labeled transferrin (50ug/ml Molecular probes) for the indicated time point and analyzed by FACS. Time 0 was collected after neutralization.

Acidification assay (pH-rodo)

Cells were pulsed 15' at 37°C with pHrodo Green Dextran (20µg/mL; Thermo scientific), washed twice with cold PBS and re-incubating at 37°C. At the indicated time point, cells were washed, incubated at 4°C and pHrodo fluorescence was analyzed by FACS.

Degradation Assay (DQ-BSA)

Cells were pulsed 10 minutes at 37°C with medium containing A647-BSA (5µg/mL; Molecular Probes). After A647-BSA pulse, cells were washed twice with cold PBS, in order to remove aspecific-bounded BSA from the cell surface. Cells were then acquired at FACS Aria III in real time at 37°C, after the first minute cells were pulsed with DQ Green BSA (5µg/mL; Thermo scientific) and acquired for 45 minutes at 37°C. DQ-BSA lysosomal degradation was measured by flow cytometry as percentage of FITC/DQ-BSA$^+$ cells, normalized on A647$^+$ cells.

Western blot

Cells were washed and lysed with a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1%SDS, 1% NP-40, and protease/phosphatase inhibitors. The lysates were centrifuged at 13,000*g* for 20 min at 4°C. Supernatants were transferred into a fresh tube and stored at -20°C. Protein concentration was determined by Bradford Pierce BCA protein assay (Thermo scientific). The supernatants were boiled for 10 min and separated by SDS-PAGE. Polyacrylamide gels were cast with Gerbu, acrylamide M-BIS 30% solution, depending on size proteins were spread on running gel from 10% to 12%.

SDS–PAGE gels and transferred onto PVDF membranes and blocked in 5% BSA 1xTBS–0.05% Tween-20 and incubated over-night at 4°C with primary antibodies (Table 2) followed by 3 washes in 1xTBS–0.05% Tween-20 and 60 min incubation with secondary antibodies. Western blots were developed with Clarity Western ECL substrate, and chemiluminescence was detected using the ChemiDoc imager (all from BioRad).

Antibody	Company	Dilution	MW (kD)	2nd Antibody
GFP	Life Technologies	1:2000	30	α-rabbit
HA	Roche	1:3000		α-rat
WASp	Santa Cruz Biotechnology	1:2000	60	α-mouse
phospho IKK α/β	Cell Signaling	1:1000	85-87	α-rabbit
total IKKα	Abcam	1:1000	85	α-rabbit
phospho NFκB p65	Cell Signaling	1:2000	65	α-rabbit
total NFκB	Cell Signaling	1:2000	65	α-rabbit
phospho ERK	Cell Signaling	1:1000	42-44	α-mouse
Total ERK	Cell Signaling	1:1000	42-44	α-mouse
Immune complex	Dako	1:1000	55	α-rabbit
PARP1	Enzo Life	1:2000	113	α-rabbit
IRF1	Santa Cruz Biotechnology	1:500	45-48	α-rabbit
Tubulin	serotec	1:25000	55	α-mouse
GAPDH	Sigma	1:70000	37	HRP conjugated

Table 2. Summary of the antibodies used in WB. Antibody name, company name, molecular weight (kD), dilutions for WB and secondary antibody are indicated.

Nuclear fractionation

$2x10^6$ cells for conditions were washed in cold PBS, lysed with cytosolic buffer containing Hepes pH 7.9 10 mM, $MgCl_2$ 1,5 mM, KCl 10 mM, DTT 0,5 mM,0,1% NP-40 and protease/phosphatase inhibitors. After 3 minutes on ice, lysate was centrifuged at 2,500 rpm for 5 min at 4°C. Supernatants were transferred into a fresh tube and stored at -20°C as cytoplasmic fraction. Pellet was washed 3 times with buffer containing Hepes pH 7.9 10 mM, $MgCl_2$ 1,5 mM, KCl 10 mM, DTT 0,5 mM. After three washes pellet was resuspended in nuclear buffer containing Hepes pH 7.9 20 mM, $MgCl_2$ 1,5 mM, NaCl 420 mM, DTT 0,5 mM, EDTA 0,2 mM and 25% glycerol. After 30 minutes on ice, we sonicate pellet for 1 minutes and pellet for 30 minutes at 15000 rpm at 4°C. Supernatants were transferred into a fresh tube and stored at -20°C as nuclear fraction

Real time PCR

Total RNA was extracted from DCs with Trizol reagent (Sigma-Aldrich) according to manufacturer's instruction. cDNA was synthesized using SuperscriptII reverse transcriptase (Invitrogen) or SuperScript VILO (Invitrogen). Real-time PCR for gene expression was performed using SoFast EvaGreen Supermix (Biorad) and specific primers listed in Table 3.

Gene	Forward	Reverse
Ifnb	TCAGAATGAGTGGTGGTTG	GACCTT TCAAATGCAGTAGATTCA
Wash	AGGTGGGGACTTGATGTCAG	AGAGAAGGCTCCTCCAGGTC
Wasp-1	GATATCGGAGCACCGAGTGG	GCAGATCCGGGTCTAGGTTG
Whamm	AGAGACATGCGAGAAGTTGC	CCTTCTAGGACCCAGCTCAT
N-wasp	AGGGTCACCAACGTGGGC	GGTGTGGGAGATGTTGTTG
Isg15	AGCAATGGCCTGGGACCTAAA	CAGACCCAGACTGGAAAGGG
Mx1	TCATCAGAGTGCAAGCGAGG	TCTGATACGGTTTCCTGTGCT
Oas1a	GGTCCAGAGTTCATGGTGGC	AACATGACCCAGGACATCAAAGG
Ifit2	GGCCTTCTGCAGTTAATGCTT	TGTGCAGCACCTCTAAGTCTCAT
Gapdh	AGAAGGTGGTGAAGCAGGCATC	AGAAGGTGGAAGAGTGGGAGTTG

Table 3. Mouse primers for qRT-PCR. Gene of interest, primer name, its 5'->3' sequence, and amplicon size.

Thermal cycle conditions were the following: 95°C for 3 minutes, 43 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. Each sample was analysed in triplicates. ΔCT (CT which the signal of the PCR product exceeds the background signal) were obtained by normalizing target genes to the reference gene. Five different genes were tested as reference genes, gapdh was identified as the more efficient. The relative expression of target genes was defined as $2\Delta^{CT} x10^3$.

Cytokine secretion

$2x10^5$ cells were stimulated with CpG-B (1µg/ml) for different times and the cytokine levels in cell culture supernatants were assessed by ELISA assay, according to manufacturer's protocols.

Cell Imaging

-<u>Fix cells.</u> To perform immunofluorescence analysis, on fixed cells, 10^5 cells with different treatment, were seeded on slides previously coated with Fibronectin (10µg/ml Sigma), followed by 30 min at 37°C for adhesion. Cells were fixed (4% paraformaldehyde), permeabilized (PBS - BSA 0,2% - saponin 0,05%), and immunoblotted after 1 hour of blocking (PBS - BSA 3% - Hepes 0,1X). Slides were incubated with the primary antibody (Table 4) 2 hours at RT. After 2 washes with PBS, slides were incubated with secondary antibody for 1 hour at RT. Fluoro-Gel II with DAPI (Electron Microcopy Science) was used as mounting medium.

Antibody	Company	Dilution	2nd Antibody
EEA1	Santa Cruz Biotechnology	1:100	α-goat
Rab5	Cell Signaling	1:200	α-rabbit
Rab11	Cell Signaling	1:200	α-rabbit
Rab7	Abcam	1:200	α-rabbit
Rab6	Cell Signaling	1:200	α-rabbit
Lamp1	BD Pharmingen	1:4000	α-rat
IRF1	Santa Cruz Biotechnology	1:50	α-rabbit
GFP	Life Technologies	1:50	α-rabbit
594/Fab	Jackson	1:800	
FITC/CpGB	InVivoGen,	1ug/ml	

FITC/phalloidin	Life Technologies	1:2000	
FITC/DQ-BSA	Life Technologies	1:500	
647/Transferrin	Life Technologies	1:500	
647/WGA	Life Technologies	1:2000	

Table 4. Summary of the antibodies used in IF. Antibody name, company name, dilutions for IF and secondary antibody are indicated.

Confocal images were acquired with LSM550 META, LSM880 META reverse microscope with a 63x objective or C1 Nikon reverse microscope with a 60X objective. Image analysis were performed using Volocity® 3D Image Analysis Software 5.5.1 (Perkin Elmer) and Fiji, ImageJ plugin (National Institutes of Health). For organelles quantification, objects were identified using a cut-off $0,1\mu m^2$ and vesicles areas were measured automatically by Volocity software.

-Time Laps. $2x10^5$ cells were seeded at 37°C on pre-coated matTek microwell dish, for 30 minutes. Time-laps movie are performed using Nikon Eclipse E-800 epifluorescent microscope supported by Andor device camera. For motility assay cells were recorded, with transmitted light, one frame every 30 seconds for 1 hour, on 20X objective. Movie are analysed using ImageJ *ManualTracking* plugin. This plugin records the x y coordinate of each cells for each frame. Data obtained by ImageJ are insert in *ChemotaxisTool* plaguing, to display motility of each cells. For endocytosis assay, cells were pulsed with 647/WGA and recorded with epifluorescence light.

Cyclohexylamide treatment

To block protein production cells and follow degradation $2x10^6$ cells were treated with 5µM Cyclohexylamide (Torcis) for different time point (3-5-7-16 hours) after this incubation time cells were lysed in RIPA buffer and analyzed by Western Blot

Actin inhibitors assay

Cells were treated with two different actin inhibitors, 25µM of CK666 (Torcis) and 0,5 µM Latrunculin A (Torcis). Optimal concentration and cell viability was previously tested in our

laboratory. Both drugs were resuspended in DMSO, used as control. From $3x10^5$ cells to $1x10^6$ were treated for 30 minutes with inhibitors. After two washes, to remove drugs, cells were used for the different experiments.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6 software. All data were reported as the mean $\pm$ SEM, the unpaired Student's *t* test was used to assess significance, unless otherwise specified.

4. Results

4.1 Enhanced innate responses upon stimulation in WKO DCs

Previous studies in our group have shown that increased production of type-I interferon by WASp KO (WKO) plasmacytoid dendritic cells (pDCs) contributes to the development of autoimmunity in Wiskott-Aldrich syndrome (Prete *et al.*, 2013). It was demonstrated that WKO pDC are intrinsically more responsive to multimeric agonist of TLR9 and constitutively secrete type-I interferon. However, the molecular and cellular basis of excessive innate activation have not been fully elucidated. I have dedicated the first part of my work to develop and validate cellular tools to address the mechanism of this phenomenon, being primary pDCs extremely difficult to obtain in large numbers and to manipulate.

4.1.1 Enhanced TLR response in WKO BM-DC.

We started by testing the responsiveness of bone marrow-derived conventional DCs (BM-DC), differentiated in complete medium IMDM supplemented with supernatant containing 30% of granulocyte macrophage colony-stimulating factor (GM-CSF) for 7 days. The differentiation and maturation profile of WT and WKO BM-DC was tested by flow cytometry to ensure that both genotype differentiate equally under GM-CSF stimulation. Cells were stained with antibodies against CD11c, CD11b (two integrins expressed on the cell surface) and maturation markers MHC class II and CD86. More than 90% of cells were CD11c$^+$/CD11b$^+$/B220$^-$, in both genotypes and up-regulation of maturation markers upon CpG-B stimulation was similar in WT and WKO cells (data not shown), indicating that WASp does not affect in vitro differentiation into DCs. To test responsiveness of WKO cDC to exogenous ligand, we stimulated cells with increasing doses of synthetic TLR9 agonist (CpG-containing phosphorothioate modified oligodeoxynucleotides) CpG-ODN class B (CpG-B) or LPS (Lipopolysaccharide from E. coli). Transcriptional activation of interferon-I (IFN-I) genes was measured after 3 or 6 hours of stimulation by real time PCR. The release of proinflammatory cytokines in the cell culture supernatant was measured by ELISA 16 hours

after stimulation. As shown in *Figure 4.1A* gene transcription and IFN-β protein production were 3 to 4-folds higher in WKO cells as compared to WT, at two different time points and both at low and high CpG-B doses. Induction of inflammatory cytokines production (TNF and IL-12p40) by CpG-B was also enhanced in WKO cells, although the increase was less pronounced than in the case of type-I interferon (*Fig 4.1B*). LPS stimulation induced a slightly increased production of inflammatory cytokines (*Fig 4.1C*) in WKO when compared to WT as control. These data indicate that WASp null BM-DCs display the same hyper-responsive phenotype to CpG-B that we had previously observed in pDCs, making it a suitable model to investigate the underlying mechanism.
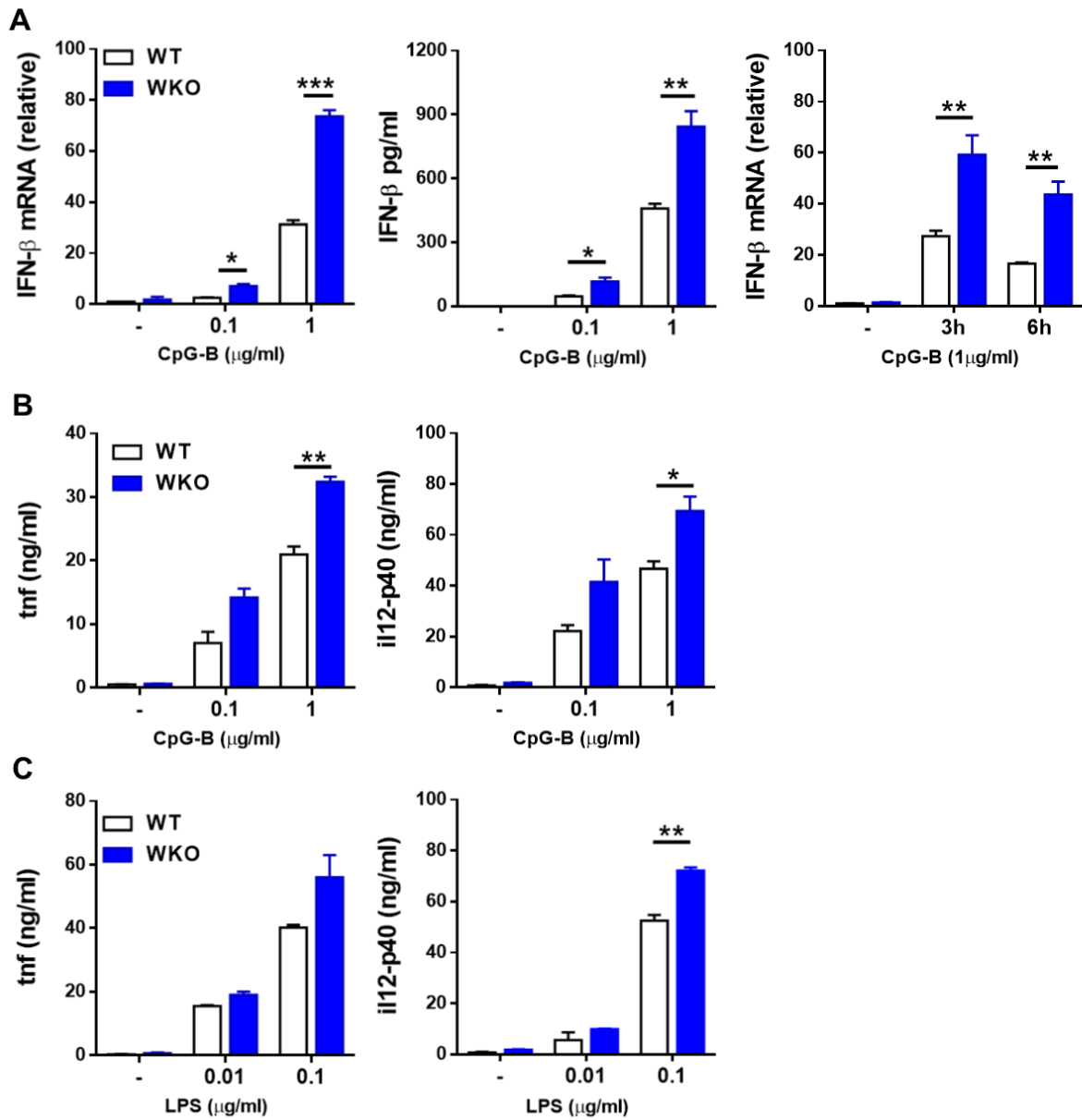
Figure 4.1 Lack of WASp enhances TLR9 and TLR4 response. Conventional DCs (cDCs) were isolated from bone marrow of WT and WKO mice and differentiated for 7 days in complete media supplemented with 30% GM-CSF supernatant. 2×10^5 cells were stimulated with synthetic TLR agonist CpG-B or LPS for different time point and doses, as indicated. (**A**) Transcriptional and protein levels of IFN-β were evaluated by RT-PCR and ELISA respectively at the doses and time points indicated. RT-PCR data are expressed as relative values on the housekeeping gene GAPDH. (B) Bars show the levels of TNF and IL-12p40 in supernatants of WT and WKO cells stimulated for 16 hours with different concentration of CpG-B (C) or LPS, measured by ELISA. All the data in the figure are means ± SEM of three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.

4.1.2 WKO cDC are hyper-responsive to endogenous TLR9 agonist

To better explore the sensitivity of WKO cells in the contest of autoimmunity we stimulated cells with dilutions of sera from Lupus prone mice (Sera Lpr) that contain multiple TLRs agonists, including complexes of autoantibodies and DNA. As shown in *Figure 4.2A*, WKO cells responded more robustly than WT. To directly focus on responsiveness of WKO innate cells to **endogenous** triggers of TLR9 and IFN-β responses, we stimulated cells with increasing doses of immune complexes (IC) formed by combining monoclonal DNA antibodies to a plasmid optimized to activate TLR9 (Henault *et al.*, 2012). Transcription of IFN-β gene was weakly induced only at maximal doses of IC (90 ng/ml) in WT cells whereas BM-DCs from WKO started to respond at low IC doses (10 ng/ml) and strongly activated gene transcription in response to 30 and 90 ng/ml (*Fig 4.2B*).
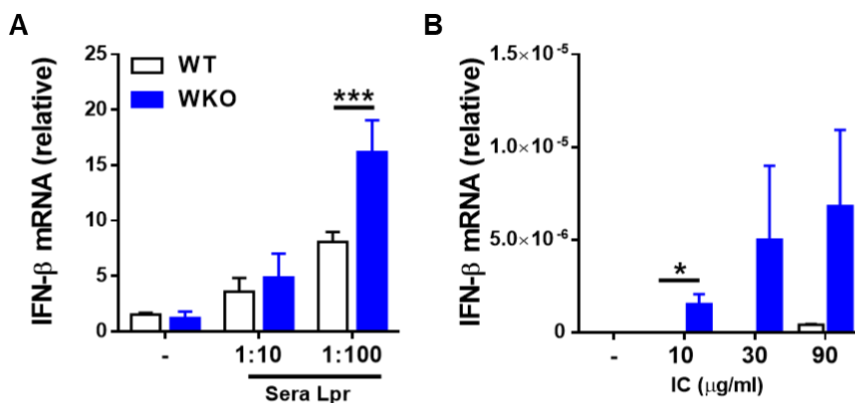
Figure 4.2. Serum from Lupus prone mice and IC induces IFN-β secretion by WKO cells. $2x10^5$ BM-DCs were stimulated with dilution of sera from Lupus prone mice (**A**) or increasing doses of IC (**B**). IFN-β gene transcript was measured after 3 hours of stimulation by RT-PCR, data are expressed as relative values on the housekeeping gene GAPDH. Values are means ± SEM of three independent experiments. *$p \leq 0.05$, ***$p \leq 0.001$.

These data indicate that WKO cells have a lower threshold for activation and are hyper-responsive to endogenous TLR9 agonist. To further support this hypothesis, we analysed transcription of interferon stimulated genes (ISGs) at steady state. Transcription of ISGs is activated by binding of IFN-I to the IFN-I receptor, via the JAK-STAT pathway. We examine four different ISGs and as shown in *Figure 4.3,* WKO DCs showed a significant induction of Mx1 and Oas1 and a mild activation of Isg15 and Ifit2, indicating constitutive transcription of these ISGs at steady state.
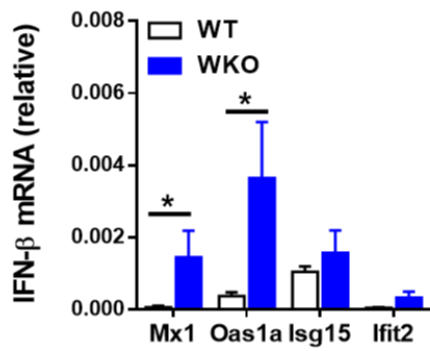
Figure 4.3 Levels of ISGs in resting cells. 2×10^5 BM-DCs were collected at steady state (in the absence of any added stimulation) and transcripts for Mx1, Oas1a, Isg15 and Ifit2 were analysed by RT-PCR. Results are presented as a relative quantification based on the relative expression levels of target gene mRNA Gapdh. Values are means ± SEM of three independent experiments. *p≤0.05.

This first set of data clearly proves the presence of an altered response to endogenous triggers of TLR9 and constitutive activation of innate pathways in WKO BM-DCs.

4.2 Generation and validation of immortalized DCs precursors.

4.2.1 Generation of Hoxb8 DCs

Since their discovery and development of systems to differentiate DCs ex-vivo from bone marrow precursors (K Inaba, M Inaba, N Romani, H Aya, M Deguchi, S Ikehara, S Muramatsu, 1992) BM-DCs allowed to address fundamental questions on their functioning, in several areas. However, this model poses some limits such the number of cells obtainable, their heterogeneity and, most importantly, their poor propensity to genetic manipulation via transfection or transduction. To overcome these limits, we decided to set in place a new method recently published in Häcker laboratory, based on retrovirus mediated expression of Hoxb8 gene under the control of the estrogen-receptor (Redecke *et al.*, 2013). This method allows to generate immortalized precursors, since Hoxb8 oncoproteins enforce self-renewal and maintain pluripotency, with potential to differentiate in myeloid cells upon removal of the expression of the Hoxb8 gene. Briefly to generate immortalized precursors, cells were collected by flushing femurs and expanded for two days under growing factors stimulation. Thereafter, BM cells were infected with retrovirus expressing estrogen regulated Hoxb8 gene (ER-Hoxb8) and cultured for one month until we obtained a population of immortalized precursors (*Fig 4.4*).
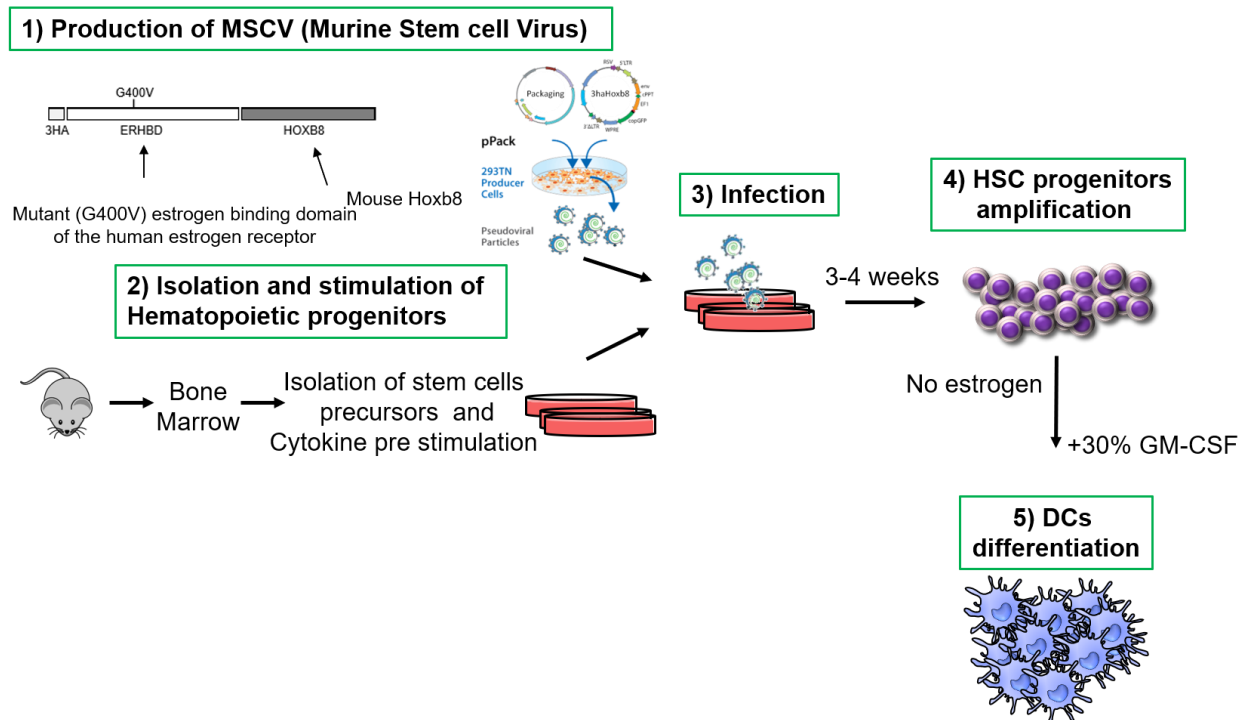
Figure 4.4 Schematically representation of Hoxb8 precursors production. 1) Schematic representation of the retroviral vector and the generation of the retrovirus in Hek cells. 2) Hematopoietic progenitors are harvested from the mouse femurs, pre-stimulated with 15% Flt3L and 30% GM-CSF supernatant to induce proliferation and initial commitment to the myeloid lineage. 3) Pre-stimulated precursors are infected at day 2 with retroviral particles and selected for the antibiotic resistance carried by the retroviral vector. 4) After 3-4 weeks colonies are amplified and 5) differentiated into DCs upon estrogen removal and addition of 30% GM-CSF for 7 days.

4.2.2. Hoxb8 potential to differentiate in DC

Once we obtained a stable population of immortalized precursors, we tested their potential to differentiate into DCs by retrieving estrogen (to inactivate Hoxb8) and by culturing the cells in the presence of 30% GM-CSF for 7 days. The resulting population was named **Hoxb8-DCs**. Differentiation in GM-CSF and analyses were conducted in parallel with cultures of primary BM-DCs. To compare differentiation and maturation upon CpG-B stimulation in fresh BM-DCs and Hoxb8-DCs we stained cells with antibodies against CD11c, CD11b, MHCII and CD86 and analysed them by flow cytometry. Immature BM-DCs (in the absence of any stimulation) are identified as CD11c$^+$ and express high level of CD11b with an intermediate expression of MHCII. As shown in *Figure 4.5A*, Hoxb8-DCs exhibited a very similar expression of CD11c and comparable CD11b and MHCII levels between Hoxb8-DC and BM-DCs. Up-regulation of maturation markers upon stimulation was also very similar in Hoxb8-DCs and BM-DCs (*Fig 4.5B*).
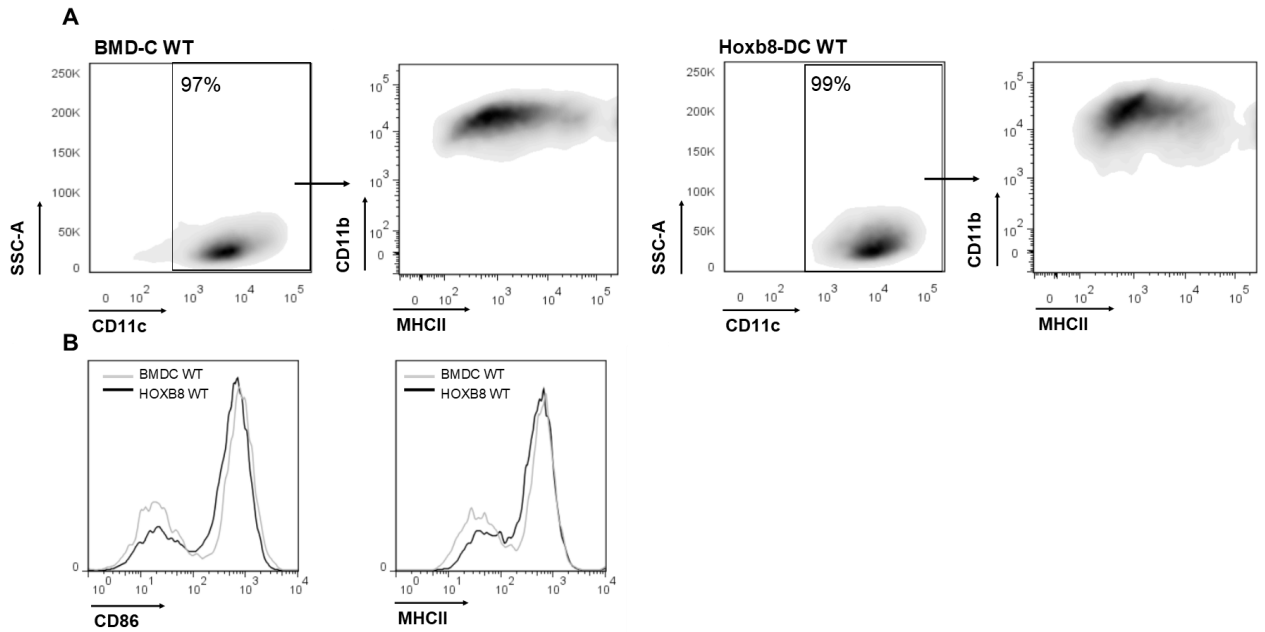
Figure 4.5 Characterization of in vitro differentiation of Hoxb8-DCs, Flow cytometry analysis of Hoxb8-DCs compared with BM-DC after 7 days of culture in the presence of 30% GM-CSF supernatant. (**A**) Immature cells were stained with Cd11c, CD11b and MHCII markers, number in quadrants indicate percentages of cells. Dot plots show representative FACS images of 3 independent experiments (**B**) Cells were stimulated with CpG B for 16 hours and stained with the maturation markers-antibodies MHC II and CD86. Histograms show representative FACS images of 3 independent experiments.

Based on this first set-up we decided to generate immortalized DCs precursors from the bone marrow of WKO animals in order to obtain a stable and homogenous source of precursors amenable to transduction. We successfully generated a line of Hoxb8 WKO precursors and tested their ability to differentiate into Hoxb8-DCs under GM-CSF stimulation. Differentiation and maturation showed similar profiles for fresh WKO BM-DCs and WKO Hoxb8-DCs, and no major differences as compared to WT Hoxb8-DCs (*Fig 4.6*)
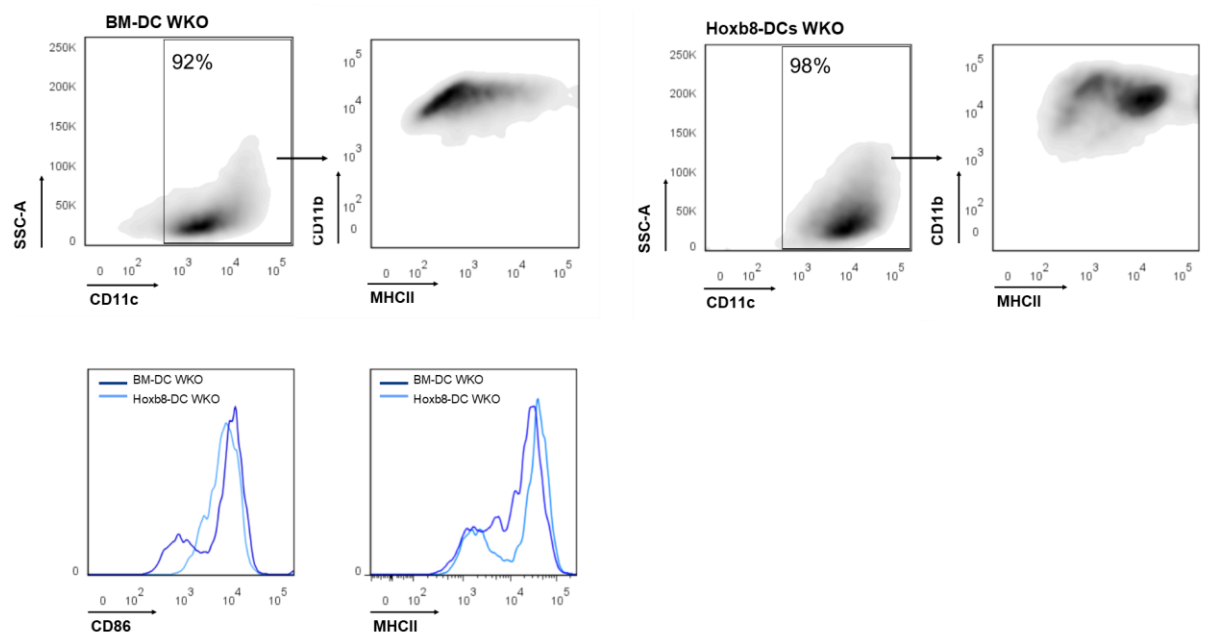
Figure 4.6 Characterization of in vitro differentiation of WKO Hoxb8-DCs, Flow cytometry analysis of WKO Hoxb8-DCs compared with WKO BM-DC after 7 days of culture in the presence of 30% GM-CSF supernatant.

4.2.3. Hoxb8-DCs maintain typical WKO phenotype

Previous analysis in our lab showed that WKO BM-DCs exhibit a flatten and extended morphology with large lamellipodia and bigger total cell area as compared to WT cells (*Fig 4.7A*). We perform the same analysis in WT and WKO Hoxb8-DCs. Cells were stained with phalloidin antibody, to mark F-actin, and total cell area was calculated by automated imaging analysis. As shown in *Figure 4.7B* WKO Hoxb8-DCs showed an increased total cell area with wide and larger lamellipodia, confirming the original WKO phenotype.
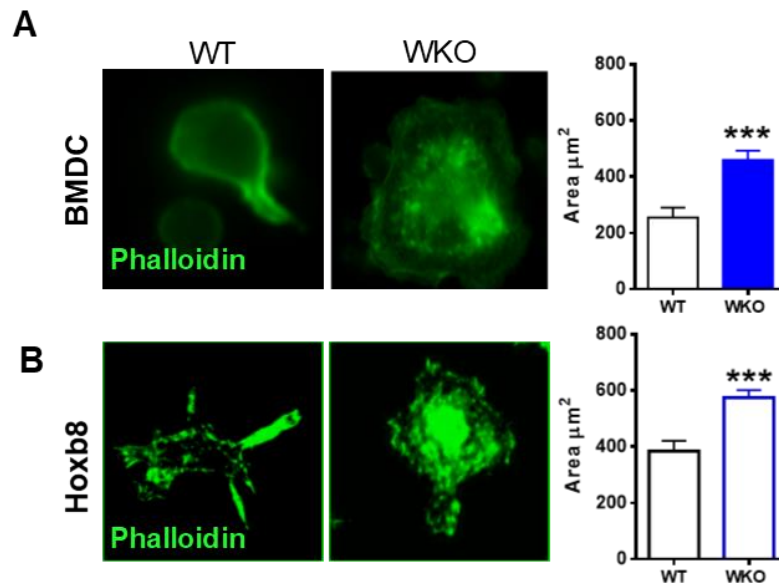
Figure 4.7. Increased cell area in Hoxb8 WKO DCs. WT and WKO Hoxb8-DCs were left to adhere on cover slips for 40 minutes, after fixation were labelled with phalloidin to mark F-actin. Images show a field on a single cell, representative confocal plane of (A) BM-DCs (from previous work in our lab) or (B) Hoxb8-DCs. Bars show the mean cell area ± SEM calculated using ImageJ software on 25/each genotype. ***p <0.0001.

Once of the central features of DCs is to start physiologic immune response and maintain tolerance. In this context a critical event for these cells is to regulate their migration and spatial localization. In 2005 the Thrasher's group showed that *in-vitro* motility of WKO BM-DC was impaired if compared to WT cells (Noronha *et al.*, 2004). Consequently, we decided to analyse the motility of Hoxb8-DCs comparing two genotypes. After CpG-B stimulation, cells were seeded on fibronectin coated surfaces and random cell motility was analysed by live imaging. Time-lapse video microscopy showed that WT Hoxb8-DCs moved rapidly across surface with persistence in directionality of movement (Euclidean distance). In contrast, WKO Hoxb8-DCs failed to establish a dominant leading edge and were unable to move appropriately. This resulted in the formation of a relatively static hyperextended cell morphology, oscillation of the cell body between each pole, and restricted translocation. Cell tracks collected by live imaging were analysed using ManualTracking ImageJ plugin and further analyzed using ChemotaxisTool to generate data on the cell trajectories on the *x y* planes, velocity and distance (*Fig 4.8*).
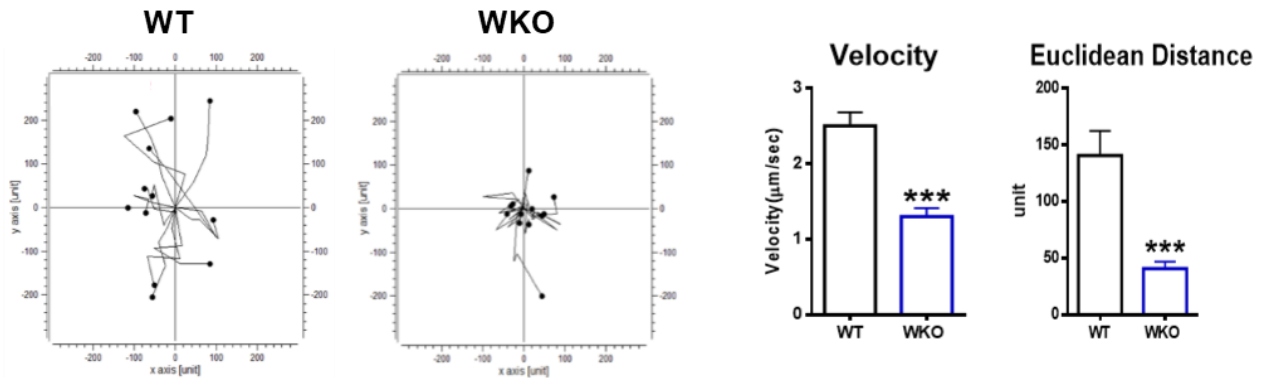
Figure 4.8 Hoxb8 WKO DCs exhibit migratory defects. WT and WKO Hoxb8- DCs were allowed to adhere to fibronectin-coated MatTeck dish before live imaging analysis. Cell movements were recorded for 45 minutes using time-lapse microscopy with frames taken at 30 seconds intervals (objective 20x). Cell tracks collected are analysed using ImageJ ManualTracking plugin and further analyzed using ChemotaxisTool to generate data on the cell trajectories, velocity and distance. Migration in Hoxb8 WKO DCs was significantly impaired. Bars show the mean ± SEM on 15/each genotype. ***, <0.001.

Thus, we concluded that WKO Hoxb8-DCs preserve the hallmark phenotype of decreased mobility and altered morphology of WKO BM-DCs.

4.2.4. Enhanced TLR response in Hoxb8 WKO DCs.

After this preliminary analysis on cytoskeletal and migratory defects in WKO Hoxb8-DCs, we test responsiveness of WKO Hoxb8-DCs to exogenous and endogenous TLR9 ligands. Transcriptional activation of IFN-β gene was measured after 3 hours of stimulation by real time PCR and the release of TNF and IL-12p40 in the cell culture supernatant was measured by ELISA after 4 and 16 hours of stimulation. As shown in *Figure 4.9A,* IFN-β gene transcription after CpG-B stimulation was 3-folds higher in WKO Hoxb8 DCs as compared to WT. Induction of IL12p40 and TNF by CpG-B was also strongly enhanced in WKO Hoxb8 DCs at two different time points (*Fig 4.9B*). Finally, transcription of IFN-β gene in response to IC stimulation was induced only in Hoxb8 WKO DC cells, whereas no induction was observed in WT Hoxb8-DCs, confirming a lower threshold of activation in WASp null cells (*Fig 4.9C*).
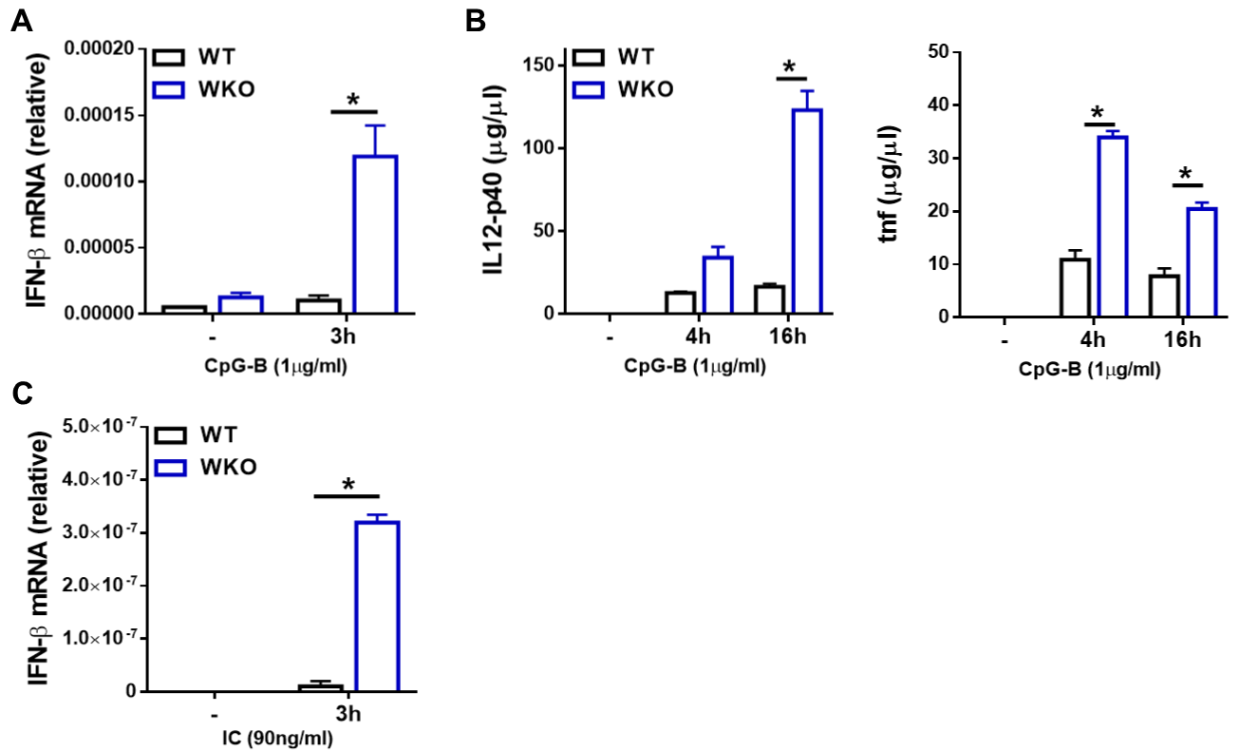
Figure 4.9 Enhanced response to TLR9 stimulation in Hoxb8 WKO DCs. 2x105 Hoxb8-DCs were stimulated with TLR9 agonist CpG-B or IC at different time points and doses, as indicated. **(A)** Bars show IFN-β gene transcript levels in WT and WKO Hoxb8-DCs stimulated with CpG-B (1μg/ml), measured by RT-PCR. Data are expressed as relative values on the housekeeping gene GAPDH. **(B)** Bars show the levels of TNF and IL-12p40 in supernatants of Hox8-DCs stimulated for 4 or 16 hours with CpG-B (1μg/ml), measured by ELISA **(C)** Gene transcript of IFN-β in WT and WKO Hoxb8-DCs after IC stimulation (90 ng/ml), data are expressed as relative values on the housekeeping gene GAPDH.All the data in the figure are means ± SEM of three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001.

4.2.5. Hoxb8 precursors are prone to transduction

A useful application of the Hoxb8 system would be to induce genes and to perform genome editing. To address this issue, we tested whether Hoxb8 precursors were prone to viral transduction. We set the conditions to infect efficiently precursors with lentiviral particles, using GFP lentiviral vector as a model. We optimized different parameters for virus production, number of cells to be infected, best amount of supernatant containing virus. As shown in *Figure 4.10*, we efficiently infected cells.
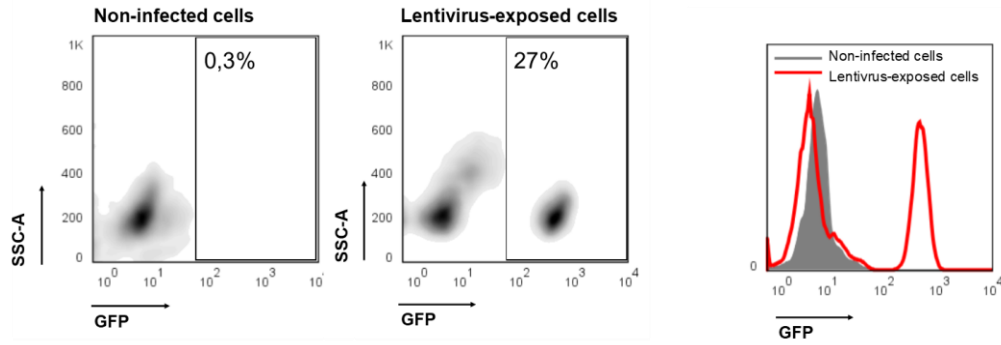
Figure 4.10 Viral transduction in Hoxb8-DCs precursors. Hoxb8 precursors were infected with lentivirus encoding GFP. Dot plots show representative FACS images cells not infected or exposed to Lentivirus. Histogram shows representative flow cytometry images of control and infected cells.

Based on these findings we concluded that, given the preservation of the phenotype of primary mutant cells and the potential to genetic manipulation, the Hoxb8-DCs system is ideal to address some key questions on the mechanism of WAS autoimmunity that we wouldn't have been able to address with fresh bone marrow DCs, as presented in the next chapters.

From here after I present data obtained using alternatively the Hoxb8 system or fresh BM-DC, depending on the experiment, as specified.

4.2.6. Generation of Hoxb8 WASp knock-out with CRIPR/CAS9

Increased ISGs expression in BM-DCs raises the question of whether *ex-vivo* hyper-responsiveness to TLR9 agonist may reflect pre-activation in the inflammatory WAS environment from which DCs arise. To formally rule out a cell-extrinsic mechanism that sensitise cells to over-respond, we generated a WASp KO cell line from WT cells using lentivirus-mediated CRISPR/CAS9 genome editing in Hoxb8 precursors. Hoxb8 WT precursors were infected with lentivirus encoding three different sgRNAs targeting first exons of WAS gene. After infection cells were first selected with puromycin antibiotics, followed by single-cell subcloning and screening of clones in which genome editing occurred. For the analysis, cells from single clones were permeabilized and stained with anti-WASp antibodies, to detect expression of intracellular protein, and analysed by flow cytometry. We isolated two clones Hoxb8WT^{CAS} (WASp$^+$) and Hoxb8WKO^{CAS} (WASp$^-$) (*Fig 4.11A*). Clones were differentiated in DCs and IFN-β gene transcript was measured after 3 hours of CpG-B

stimulation by real time PCR. As shown in *Figure 4.11B*, WASp⁻ induced a higher IFN-β response as compared to those produced by WASp⁺ precursors.

These data prove that activity of WASp in DCs acts as a negative cell-intrinsic regulator of TLR9 ligand.
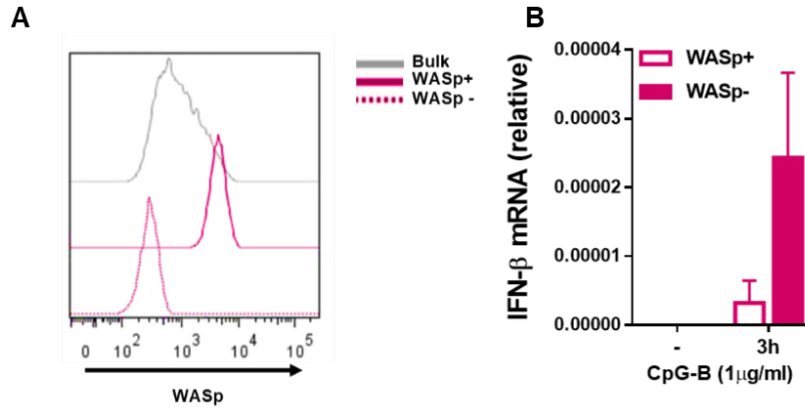

Figure 4.11 Genome editing on Hoxb8 WT cells. WT Hoxb8 precursors were infected with lentivirus encoding three different sgRNA for WAS gene and isolated by single-cells cloning. (**A**) Histogram show representative flow cytometry images of bulk population after infection and the two sub clones Hoxb8 WAS⁺ and WASP⁻ precursors. (**B**) Gene transcript of IFN-β in Hoxb8 WAS⁺ and WASP⁻ DCs 3 hours after CpG-B stimulation, data are expressed are means ± SEM of three independent experiments, as relative values on the housekeeping gene GAPDH.

4.2.7. Analysis of intracellular WASp distribution using lentiviral mediated over-expression of WASp-GFP in Hoxb8 cells.

In the last years nucleation promoting factors (NPFs) have been extensively studied (Takenawa and Suetsugu, 2007). Among this family, WAVE proteins are involved in the formation of lamellipodia and membrane ruffles, WHAMM facilitates transport along the biosynthetic pathway and is required to maintain Golgi morphology (Campellone *et al.*, 2008). JMY seems to be involved in Arp2/3 complex-mediated leading edge protrusion and cell motility (Rottner, Hänisch and Campellone, 2010). Actin polymerization by WASH is important to control shape and maturation of endosomes, regulating fission of these organelles (Derivery *et al.*, 2009). N-WASP is essential for actin assembly at the surface of endo-membranes and the hematopoietic protein WASp is implicated in various actin based processes such as podosome formation, phagocytosis and chemotaxis (Benesch *et al.*, 2002; Burianek and Soderling, 2013). However, the possibility that hematopoietic WASp may play a role in endosomal trafficking was poorly explored so far. To address this question, we analysed the subcellular distribution of WASp. Initial attempt using antibodies to WASp

failed as the signal was too faint. Thus, we decided to generate a line stably expressing eGFP-WASp using Hoxb8 and lentiviral transduction. WKO Hoxb8 precursors were transduced with eGFP-WASp (lentiviral vector from Thrasher group). Cells were sorted two weeks after infection to obtain a homogenous population of cells expressing eGFP-WASp. After sorting we check the purity of sorted cells by flow cytometry (*Figure 4.12A*). Cells were differentiated into DCs and labeled with antibodies to GFP (to enhance eGFP-WASp signal) and markers of early endosome (EEA-1), lysosomes (LAMP1) and Golgi (Rab6). As shown in *Figure 4.12B*, the majority of WASp labeling is found in peripheral structures near the plasma membrane, as expected. However higher magnification images showed a well a fraction of WASp signal localized around EEA1 marker. Colocalization with LAMP-1 instead was limited. Interestingly, we found a strong association to the Golgi compartment. Consistently, the Pearson correlation coefficient (PCC) was high for EEA-1 and Rab6 and lower for LAMP-1.
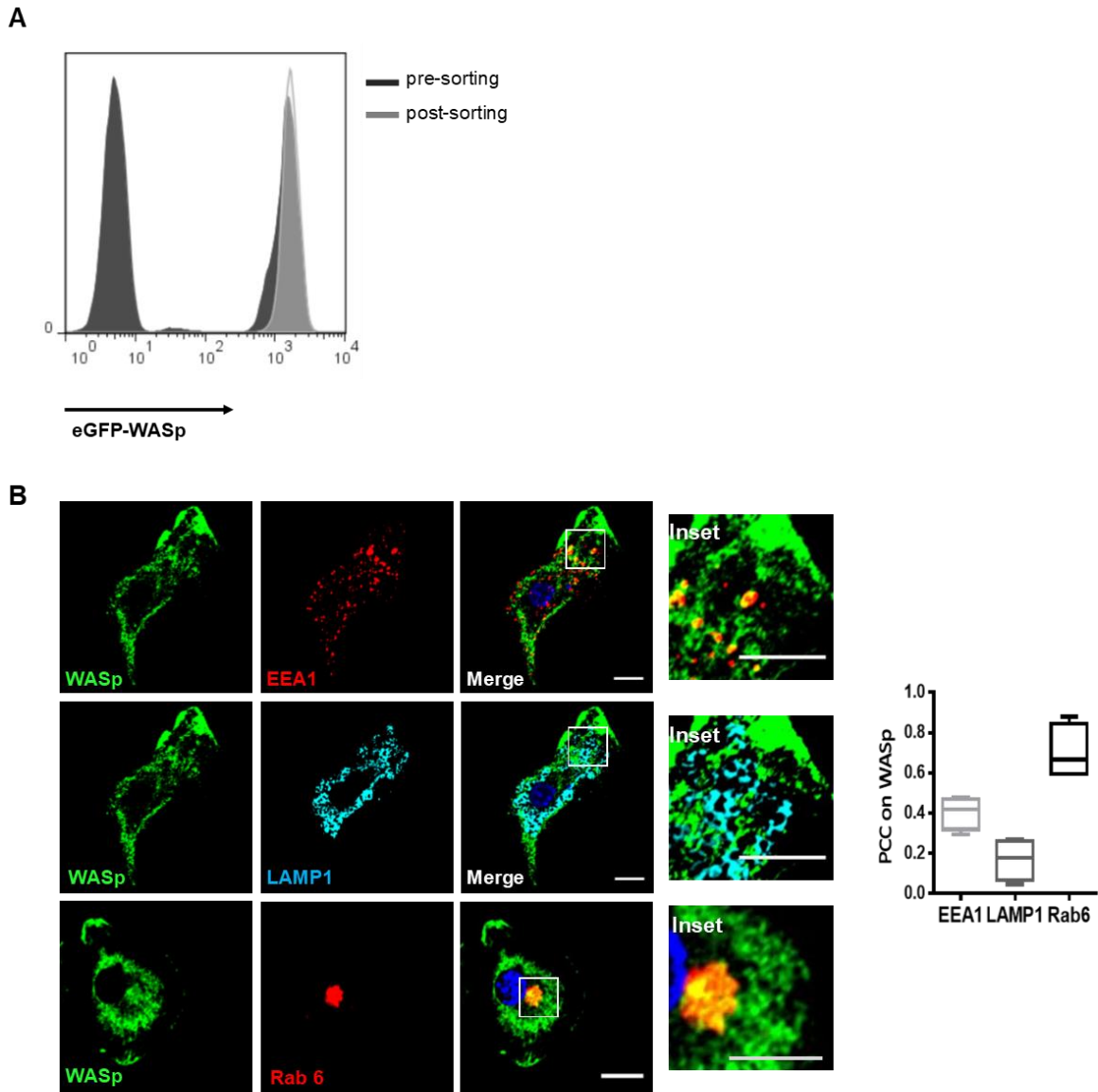
Figure 4.12 WASp intracellular localization. WKO Hoxb8 precursors were infected with lentivirus encoding eGFP-WASp, (**A**) cells obtained by lentiviral infection were sorted by FACS Aria III to obtain a homogenous population expressing eGFPWASp. (**B**) $1X10^5$ cells were left to adhere on cover slip for 40 minutes, after fixation were labelled with EEA1 (red), LAMP1 (light blue) and WASp (green). Images show a field (63 x magnification) on a single cell representative. Bars show the Pearson correlation coefficient (PCC) between WASp and marker of interest ±SEM n=20.

4.3 Structural analysis of the endocytic pathway

4.3.1 Lack of WASp affects early endosome morphology and distribution

Our previous results showed that WASp localizes also around endosomes. Therefore, we moved to analyse endosome structure in WT and WKO DCs. We labelled early endosomes, with EEA1 marker, in not stimulated Hoxb8-DCs and BM-DCs. Individual cells were acquired by confocal microscopy to quantify area of EEA1$^+$ vesicles. We found that WT BM-DCs contained several well-defined rounded vesicles with an average area of 0,45 μm^2, that were distributed uniformly within the cell. In contrast, in WKO BM-DCs we found a significant increase in size in the early endosome. WKO BM-DCs often contain a large vesicle (0,7 μm^2) at one pole of the cell, suggesting a clustering of several fused endosomes (*Fig. 4.13A*). As shown in *Figure 4.13B*, also WKO Hoxb8- DCs present EEA1$^+$ vesicles that are larger than control cells.
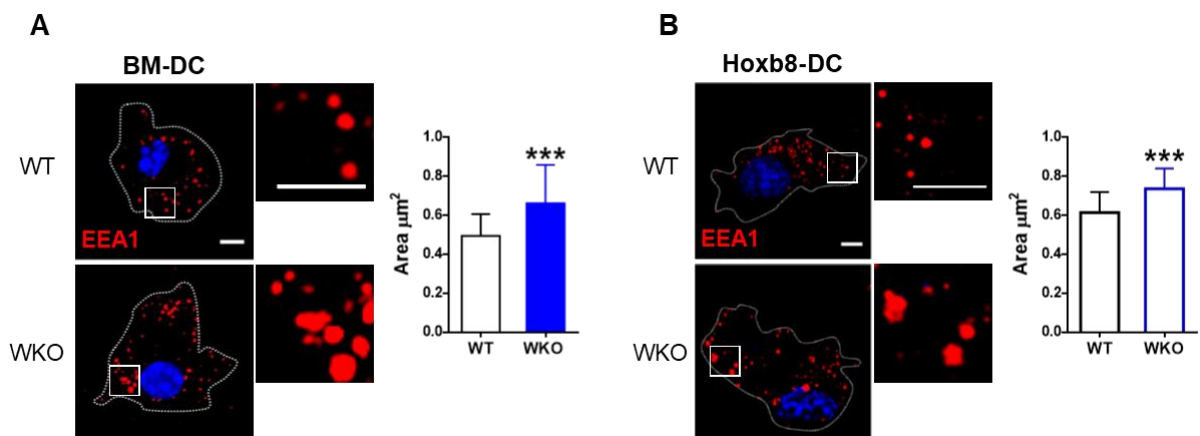

Figure 4.13 Enlarged endosome in WKO DCs. 1x10^5 BM-DCs and Hoxb8-DCs were stained with EEA1 antibody. Images show representative single confocal planes. (A) Endosomes area was quantified on 35 WT and 32 WKO BM-DCs. (B) Endosomes area was quantified in 38 WT and 27 WKO Hoxb8-DCs. Scale bar 10 μm and 5 μm on inset. Bars show endosome area mean ± SEM ***, p<0.001.

Given the homogeneity between Hoxb8-DCs and freshly isolated BM DCs, we used in forthcoming experiment either of the two models without distinction and refer simply to DCs.

To confirm our observation, at the ultrastructural level, we performed analysis on endosome size with electron microscopy (EM). DCs were fixed, and sent to Utrecht, at the University Medical Center, to be analysed. Our results on endosome enlargement were confirmed also by EM analysis (*Fig 4.14*).
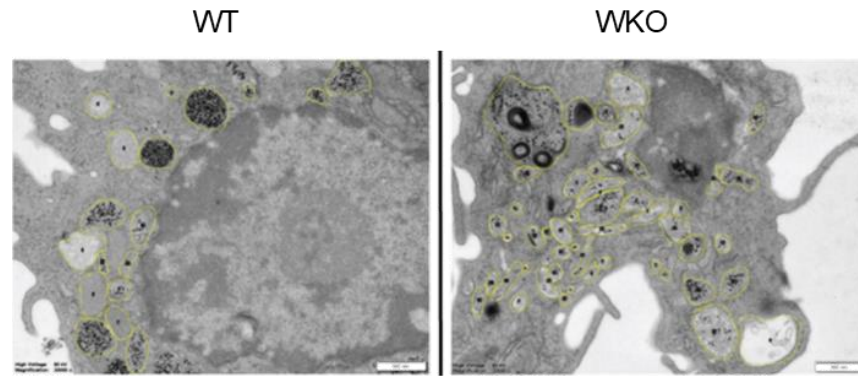
Figure 4.14 Morphologies of endosomes at the ultrastructural level. Electron micrographs of endosome located in WT and WKO DCs. Endosome are surrounded by yellow line.

From the quantification of endosomes size, we noticed that EEA$^+$ vesicles were differently distributed in WT and WKO cells. We decided to perform a deeper analysis on endosome distribution. DCs were stained again with antibodies to EEA1 and we calculated the distance of each vesicle from the nucleus. This analysis showed that endosomes in WKO DCs are spread in the cytoplasm and remain more peripheral than in control cells in which EEA$^+$ organelles are more perinuclear (*Fig 4.15*).
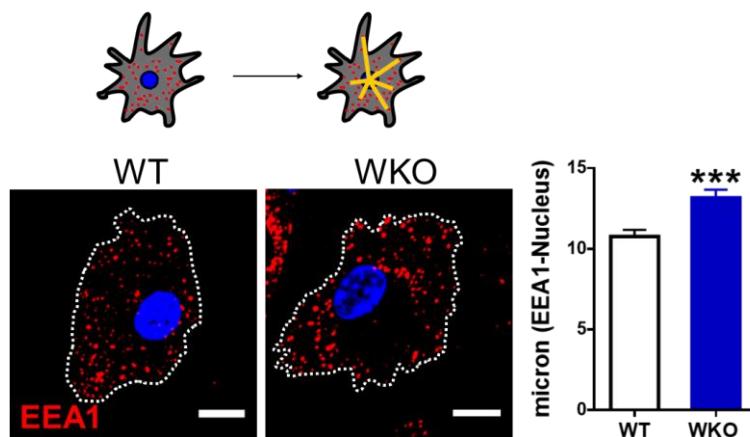

Figure 4.15 Peripheral endosome in WKO cells. 1×10^5 DCs were stained with EEA1 antibody and DAPI to stain the cell nucleus. Images show representative single confocal planes. To measure the distance of each endosome from the nucleus the length of the segment between the centroid of the endosomes and the centroid of the nucleus was calculated using ImageJ. Data were collected from 28 WT and 29 WKO cells, scale bar 10 Bars show distance between endosome and nucleus in micron mean ± SEM ***, $p < 0.001$.

Newly formed endocytic vesicles are transported away from the actin-rich cell periphery before docking to the microtubule transport system (Huotari and Helenius, 2011).

These data suggest that endosomal sorting and maturation is perturbed in WKO DCs.

4.3.2 Tracking endocytosis in live imaging.

It's known that endosomes are motile, and their movements strongly linked to their stage of maturation and function. To follow the dynamics of endocytosis, we performed a live imaging assay. Cells were loaded with wheat germ agglutinin (WGA) Alexa 647 conjugated, (WGA/A647) that selectively binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues on plasma membrane. Upon binding WGA is progressively internalized and transported along the endocytic pathway marking different stations along the pathway and ending up in lysosomes after 2-3 hours (Kotsias *et al.*, 2015). Cells were loaded with WGA, washed and immediately recorded for 40 minutes, every 30 seconds. In WT cells the WGA/A647 signal was initially distributed homogenously in the plasma membrane. Small vesicles begun to pinch off the membrane after 5 min and moved quickly toward the cell center. We observed striking differences in the appearance of vesicles in WKO DCs. Large vesicles formed underneath the membrane and remained anchored at membrane for up to 30 minutes. Only at later time points some large WGA positive structures was seen detaching for the membrane and moving toward the cell center. Figure *Fig 4.16* shows a representative example of this behavior.
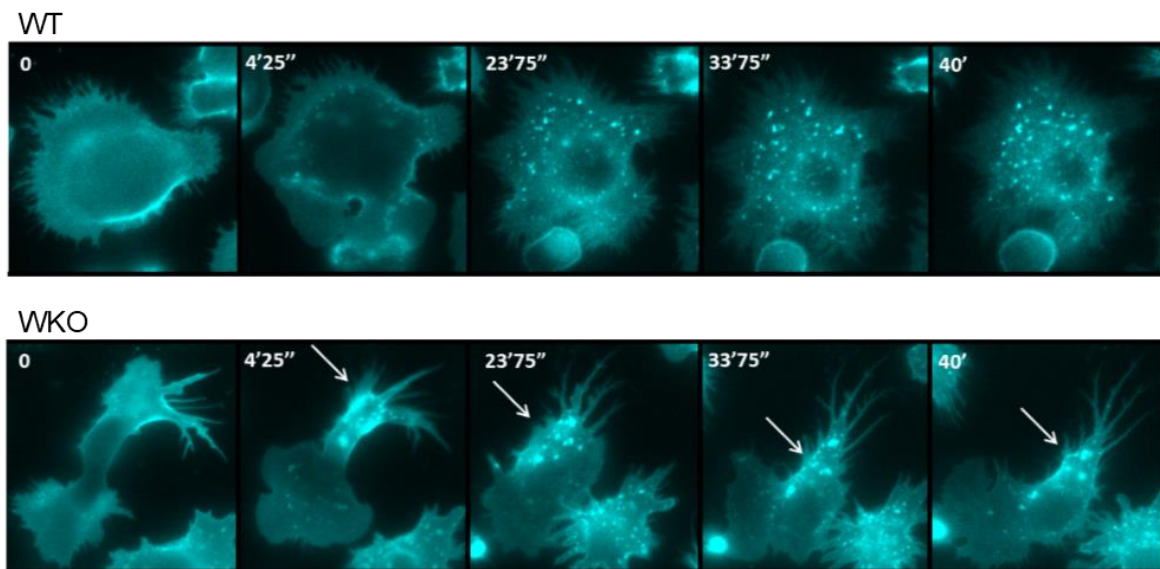
Figure 4.16 WGA/A647 internalization to mark endocytosis. $2x10^5$ DCs were seeded for 30 minutes in a Nikon chamber at 37C° with CO_2 5% on MatTek microwell dish. After adhesion time cells were pulsed for 5 seconds with WGA/A647 5µg/ml. After two washes in PBS and warm complete media was added. Cells were recorded in epifluorescence with a 60X magnification for 40 minutes, one frame every 30 seconds. In figure are shown 5 representative frames for each genotype.

A recent study, based on multiparametric images analysis, demonstrates that location of endosome in the cytoplasm, measured as distance from the nucleus, is an important parameter that influences cargo contents consistent with the spatio-temporal progression of endosome maturation (Collinet *et al.*, 2010). Our results strongly suggest that actin assembly by WASp is important for the regulation of endosomal shape, distribution and maturation.

4.3.3 Distribution of Rab proteins in WT and WKO DCs

Is well established that dynamic trafficking of intracellular membranes is governed by a complex molecular machinery that regulates different steps like vesicle budding, movement, docking, and fusion. Rabs are localized to the cytosolic face of specific intracellular membranes and act as regulators of distinct steps in membrane traffic pathways (Stenmark and Olkkonen, 2001; Li *et al.*, 2016).

To better understand the role of WASp in intracellular endosome trafficking and maturation, we moved to study the distribution of key Rab proteins in WT and WKO DCs. We stained DCs with four Rabs proteins associated with various endosomal compartments. Rab5 localizes to early endosomes where it promotes recruitment of Rab7 and maturation of early endosomes to late endosomes, Rab7 serving to regulate transport from early to late endosomes and the first step to lysosome (Feng, Press and Wandinger-Ness, 1995; Vanlandingham and Ceresa, 2009). Rab11 associates mostly with recycling endosome and regulate protein recycling and the exocytosis at the plasma membrane (Takahashi *et al.*, 2012) and Rab6 is associated to intra-Golgi transport, either acting as a positive regulator of retrograde transport or an inhibitor in anterograde transport (Martinez *et al.*, 1994). Area of organelles detected from each staining were examined. In WKO DCs the area of Rab5, Rab7 and Rab6 organelle were significantly increased as compared with WT, with the presence of large aggregates resembling fusion of multiple vesicles. In contrast analysis of recycling organelles (Rab11) didn't show any differences, in size, between two genotypes (*Fig 4.17*).
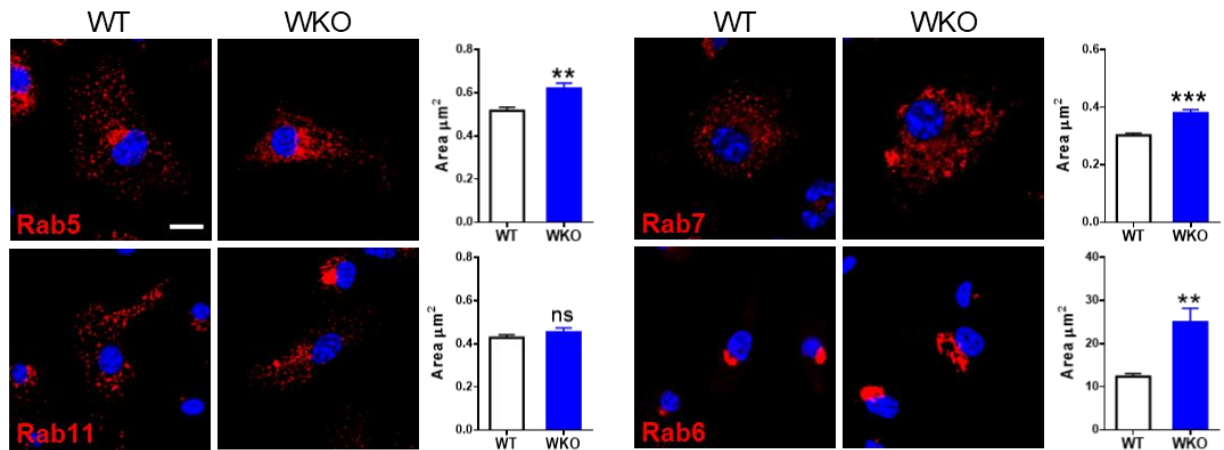
Figure 4.17 Rabs analysis. 2×10^5 DCs were seeded for 30 minutes at 37°C on pre-coated coverslip and stained with different endo-lysosome marker: Rab5, Rab7, Rab11 and Rab6. Images show representative single confocal planes from 20 WT and 20 WKO cells. Scale bar represents 10 µm. Bars are means ± SEM. **p≤0.01, ***p≤0.001.

Together these analyses demonstrate that cells deficient for WASp present aberrations in the structure of endocytic pathway and suggest a role for WASp in endosomal fission/sorting or docking of vesicles on microtubules for progression and maturation.

4.4 Functional analysis of the endocytic pathway

Experiments in the previous section suggest that endocytic structures are blocked in maturation, fission or in recycling. To directly test this hypothesis, I performed functional assays by flow cytometry analysis using different endocytic probes.

4.4.1 Analysis of recycling in WKO DCs

To investigate possible effect of lack of WASp on recycling from sorting endosomes to the cell surface we analysed transferrin (Tf) recycling using fluorescently conjugated Tf and flow cytometry. Tf is an iron-binding protein that favours iron-uptake in cells. Iron-loaded Tf binds the Tf receptor (TfR) and enters into the cells. Inside the cell, Tf is trafficked to early endosomes, releases iron in an acidic compartment, and is then sorted to recycling endosomes to be taken back to the cell surface. To monitor the kinetic of this process I have tested several conditions and I have found the optimal conditions to measure recycling in DCs. Briefly, cells were starved for 30 minutes followed by a pulse with Tf/647 for 1 hour to reach equilibrium. After a quick wash with an acid buffer, to remove surface bound Tf, cells

were immediately acquired (t=0) or incubated at 37°C for different time points in the presence of an excess of not-labelled Tf. At each time point the reaction was stopped by placing cells at 4°C and analysed on a flow cytometer set at 4°C, to avoid further recycling. Recycling correspond to loss of mean fluorescence intensity (MFI) with respect to t=0 (100%), WT DCs started to recycle within the first 5-10 minutes of incubation at 37°C and after 40 minutes, 24,5% ($\pm$9,8 SD) of internalized Tf had recycled (*Fig 4.18A-B*). In contrast, fluorescence remained stable for 10-15 min in WKO DCs and only begun to decrease between 20 and 40 min, and reached values of 16,5% ($\pm$5,6 SD) at the end of the assay. Thus, Tf recycling is significantly slowed down in WKO cells. Microscopy images captured at three different time point (*Fig 4.18C)* confirmed the presence of more abundant intracellular Tf vesicles in WKO cells after 10 and 40 min of chase.
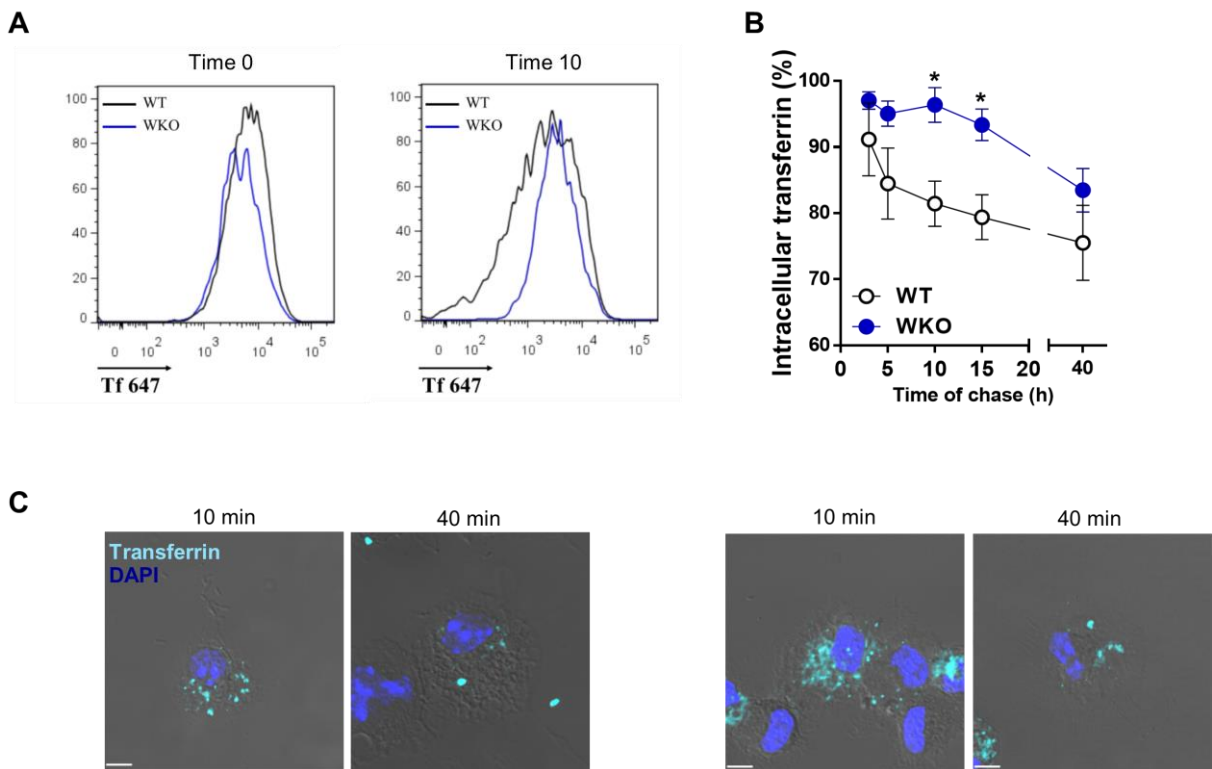
Figure 4.18 Transferrin uptake and recycling (**A-B**) WT or WKO DCs were loaded with Tf/647 1 hours at 37°C and then acid washed at 4°C. After a shift to 37°C for the indicated times, intracellular Tf was quantified by flow cytometry. A, Intracellular Tf is expressed histogram at two different tie points. B, Intracellular Tf is expressed as a percentage of the initial intracellular Tf. mean $\pm$ SEM of Tf MFI, normalized on t0 of three indipendent experiments. Significance was determined by unpaired t test with *p$\leq$0.05. (**C**) Images show representative single epifluorescence plane of WT and WKO cells at three different time point.

4.4.2 Analysis of endosome acidification in WKO DC

One important step in endosome maturation, is acidification of luminal part of organelle. Decrease in pH provides a better environment for hydrolytic reactions and it is also essential for membrane trafficking, sorting and routing of cargo. The differences in extracellular compartment and early endosome internal pH provides the asymmetry needed to allow receptors to bind ligands in one compartment and release them in the other. Therefore, the progressively decreasing pH in the endocytic pathway can provide incoming cargo a 'sense' as to their location within the pathway (Huotari and Helenius, 2011). To explore levels of early endosome acidification, we tested intracellular pH. Cells were incubated with pHrodo green dextran, a pH-sensitive probe that is internalized by endocytosis and emits fluorescence only in acidic organelles, for 15 minutes. After quick wash cells were incubated for different time points at 37°C and stopped at 4°C for analysis of the MFI by flow cytometry. The fluorescent signal started to appear after 20 min of incubation at 37°C, indicating that the probe has reached an acidic compartment and kept on increasing for the following 80 minutes. In WKO cells we observed a trend towards lower MFI values but given the variability among experiments the differences were not statistically significant (*Fig 4.19A-B*).

As control of the probe sensibility, we treated cells with NH$_4$Cl, to block endosomal acidification. Cells were pre-treated for 30 minutes at 37°C with NH$_4$Cl and then pulsed with pHrodo green dextran for 90 minutes in presence of NH$_4$Cl. In *Figure 4.19C* are shown data, of two independent experiment, proving that the treatment abolishes the activation of the probe.
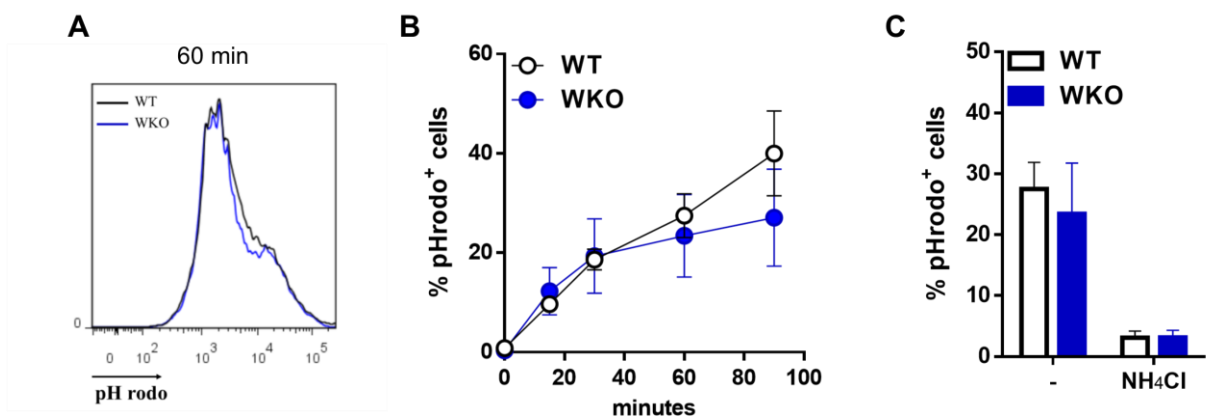

Figure 4.19 Similar endosomal acidification in WT and WKO DCs. WT or WKO DCs were pulsed with pH-sensitive pHrodo Green dye (20μg/ml) 15' at 37°C. Washed twice with cold PBS and blocked at 4°C (t0) or incubated 10', 30' 60' and 90' at 37°C to allow internalization. (**A**) Histogram represent pHrodo internalization

after 60 minutes. (**B**) Data are presented as acidification index that represent mean ± SEM of pH-rodo$^+$ cells of three indipendent experiments. (**C**) Cells were treated as previously described for 60' with or without NH$_4$Cl to block organelle acidification. Cells treated with NH$_4$Cl are not positive for pH-sensitive. Data presented as mean ± SEM of 2 independent experiments.

These preliminary results showed no clear differences between two genotypes, so we moved to a further functional assay to measure functional degradation.

4.4.3 Analysis of lysosome degradative activity in WKO DCs

Lysosomes are the terminal end of the endocytic pathway; the acid environment is required to activate proteases and hydrolases that degrade the cargo delivered to these compartments. This process of cargo delivery and degradation by endo-lysosomes compartment is a tightly regulated process.

We reasoned that the observed alterations in morphology and recycling might be linked to a decrease in maturation of endosomes into degradation competent organelles. To directly test this hypothesis, we incubated cells with DQ-BSA (Dye Quenched-Bovine Serum Albumin), a self-quenched probe that emits bright fluorescence only upon proteolytic cleavage in endosomes. Cells were loaded with DQ-BSA and degradation activity was evaluated by flow cytometry in real time at 37°C for 45 minutes. To control for uptake cells were pre-pulsed with A647-BSA and degradation was normalized on A647-BSA$^+$ cells. The increase in fluorescence (that indicates degradation) followed a similar trend in both genotypes, but the intensities were significantly higher in WT cells as compared to WKO cells at all time points tested (*Fig 4.20A*). A higher intracellular fluorescence in WT was confirmed by confocal microscopy. (F*igure 4.20B).*
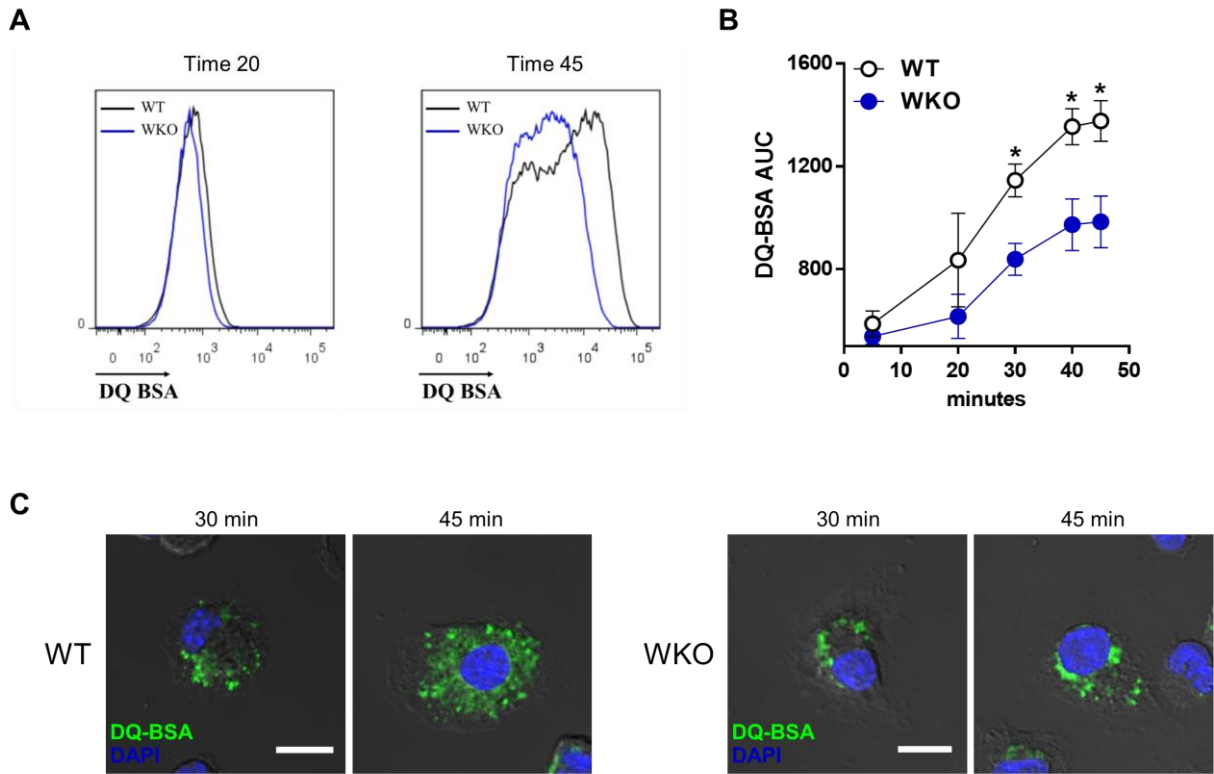
A

B

C

Figure 4.20 Impairment degradation in WKO cells. WT or WKO DCs were pulsed with A647-BSA (as control of uptake) 10' at 37°C. Cells were then washed and FITC/DQ-BSA was added before flow cytometry analysis, as an index of degradation. (**A-B**) Sample were acquired in Real-Time for 45' at FACS ARIAIII. Data are normalized on A647-BSA uptake. A, FACS histogram represent degradation at two different time point. In B data are represent as the area under the curve (AUC). Data are mean ± SEM of 3 independent experiments. Significance was determined by unpaired t test with *p≤0.05. (**C**) Images show representative confocal plane of WT and WKO DCs at two different time points of chase after DQ-BSA pulsing.

Taken together these results strongly suggest the importance of WASp in controlling maturation or endocytic organelles into lysosomes and suggest a possible impact of WASp deficiency on trafficking of TLR9 and its ligands.

4.5 TLR9 receptor

It is known that TLRs intracellular localization is highly regulated. UNC93B1 regulate exit from ER and facilitates incorporation of TLR9 in COPII vesicles (Barton, Kagan and Medzhitov, 2006; Subramanian *et al.*, 2006). Several studies in recent years have begun to shed light on the mechanisms of TLR9 trafficking and on the importance of its localization for signal initiation and termination (Akbar et al. 2015).

Although the trafficking steps leading to receptor activation have been defined in most details (ER-endosome translocation and cleavage to generate a functional receptor), the importance of endosomal maturation for attenuation and termination of the signal is less understood. Few studies suggest that after ligand binding TLR9 is degraded in lysosomes (Chuang and Ulevitch, 2004).

4.5.1 TLR9 overexpression in Hoxb8 precursors.

Results presented in the previous sections show that in WKO DCs alterations in endocytic compartments lead to aberrant endo-lysosome function. Therefore, we hypothesized that these defects may impact on the dynamics of TLR9 trafficking, localization and degradation.

Biochemical analysis on TLR9 in DCs are limited since commercial antibody are not able to detect endogenous receptor. Thus, we used Hoxb8 precursors to generate stable lines of WT and WKO DCs expressing TLR9 tagged to HA. The expression plasmid, a kindly gift from Barton laboratory, was obtained by insertion of TLR9-HA (at the C-terminal end) in a MSCV2.2 IRES GFP retroviral plasmid. The GFP on a separated transcriptional unit allows to select for cells expressing TLR9-HA fusion protein and to normalize for the levels of transduction. Cells were infected as previously described and one week after infection we check the efficiency of transduction. Cells were fixed and permeabilized to visualize intracellular HA and GFP by flow cytometry. As shown in *Figure 4.21A*, efficiency of transduction was not very high, 7,8% in WT precursors and only 3,5% in WKO. To obtain a homogenous population cells were expanded, to obtain a large number of cells, and sorted for the same MFI. The purity of both population after sorting was higher than 99% (*Fig 4.21B).*
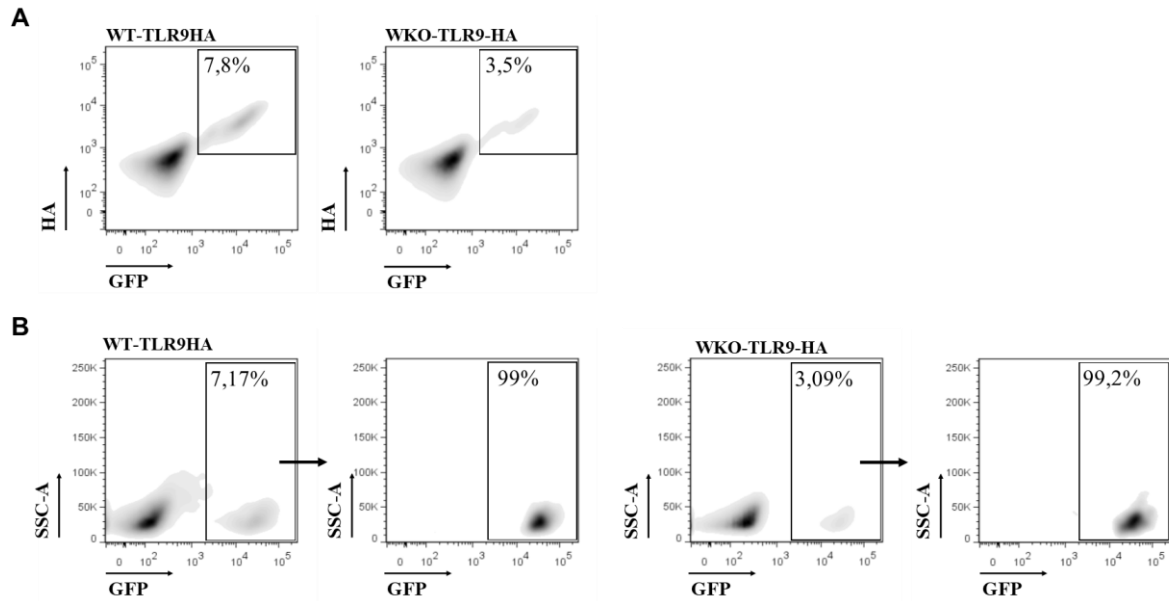
Figure 4.21 TLR9 overexpression in Hoxb8 precursor. Flow cytometry analysis of Hoxb8 cells after TLR9-HA retroviral infection (**A**) Cells were stained with rat-anti-HA antibody followed by secondary anti rat Alexa647 and analyzed also for expression GFP. Positive number in quadrants indicate percentages of cells. (**B**) 10x10^{6} cells were sorted for GFP+ population. Histograms show representative FACS images

4.5.2 Analysis of TLR9 expression in Hoxb8 DCs.

Although TLR9 ligand recognition occurs in endo-lysosomes, it has been reported that most TLR9 resides in the endoplasmic reticulum (ER), traffics across Golgi and cell surface to reach endo-lysosome signalling compartments (Ewald *et al.*, 2008; Onji *et al.*, 2013). As mentioned before, TLR9 strategic subcellular localization is important to discriminate between self and non-self. Moreover, a further important regulatory checkpoint to control TLR9 receptor activity is the cleavage of its ectodomain (Ewald *et al.*, 2008). The cleavage requires specific enzymes that reside and become activated only in endosomes (Ewald *et al.*, 2011).

To evaluate whether WASp might control TLR9 production and cleavage, we firstly analysed production of TLR9-HA protein in resting cells. Cell lysates were analysed by immunoblotting with an anti-HA antibody to detect TLR9 and an anti-GFP antibody as loading control.

As shown in *Figure 4.22A* in total cell lysates both fractions of receptors, full length (FL, 150 kDa) and cleaved (80 kDa) are visible in not-stimulated cells, indicating that a fraction of the receptor constitutively traffics to endosomes. The ratio between FL and

cleaved receptor as well as the total TLR9-HA levels normalized on GFP do not differ substantially between the two genotypes (*4.22 A,B*), indicating that synthesis and cleavage into a functional receptor is not regulated by WASp expression.
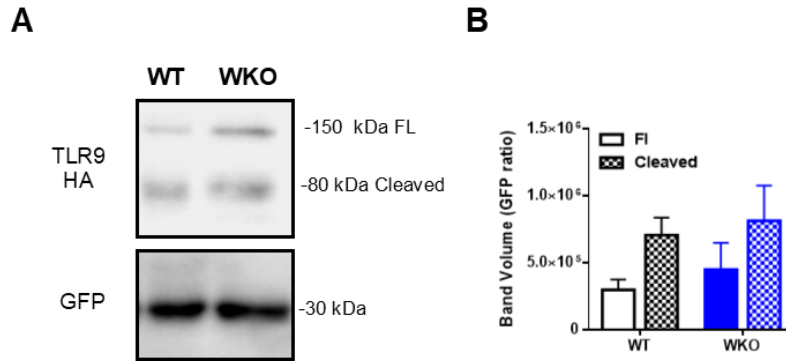

Figure 4.22 Biochemical TLR9 detection in DCs. (**A**) The whole cell lysates of $5x10^5$ DCs were analysed by Western blot with an anti-HA and anti-GFP antibodies and detected with chemidoc touch BioRad (**B**) Bars show the band volume mean ± SEM of three independent experiments normalized on the total GFP intensity used as loading control. Quantification is performed using ImageLab from BioRad.

4.5.3 Inhibition of protein synthesis induces delay in TLR9 degradation in WKO DCs.

The fate of TLR9 receptor after signalling in endosomes is not fully understood. Few studies have formally addressed the pathways for TLR9 degradation. An old report suggests that ubiquitination by TriadE3 affects signalling by TLR9, possibly via degradation (Chuang and Ulevitch, 2004). Rab7b was implicated in negative regulation of TLR9 by favouring delivery to lysosomes for degradation (Wang *et al.*, 2007).

Based on our data, we speculated that the increase in inflammatory response in WKO cells may be due to retention of functionally active TLR9 in endosomal compartments and delayed degradation. To test this hypothesis, we treated WT and WKO DCs with cycloheximide 5µM (CHX), a potent protein synthesis inhibitor. Aliquots of cells were collected at specific time points (0-3-5-7-16 hours) and total TLR9 protein was assessed by western blot (*Fig 4.23A*).
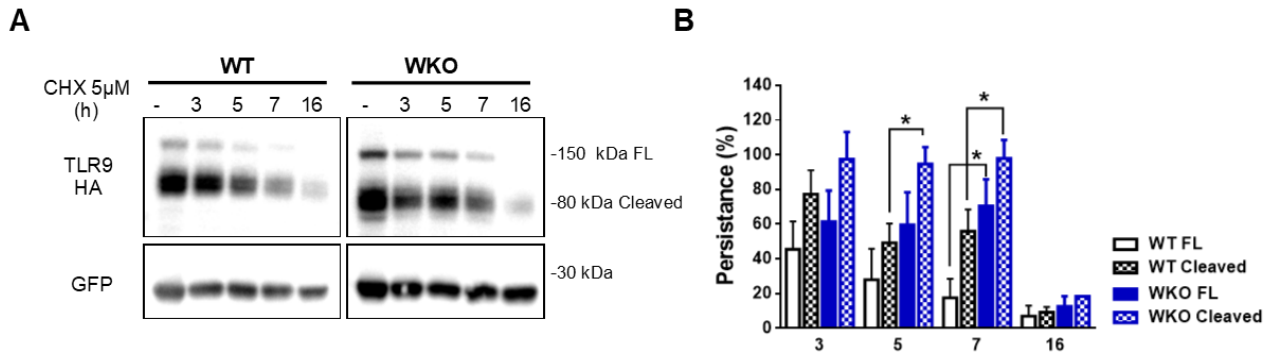
Figure 4.23 WKO DCs have reduced TLR9 protein degradation. (**A**) Representative Western blot of TLR9 degradation in cells treated with 5µM CHX for different time points, as indicated. The whole cell lysates were detected with an anti-HA and anti-GFP antibodies. (**B**) HA expression is normalized on GFP and band persistence (%) is related to time 0. Bars show mean ± SEM of three independent experiment *p≤0.05.

In WT DCs we observed a steady decrease in FL TLR9 receptor with only ≈20% of the initial material present after 7 hours of chase. Disappearance of the cleaved part was slower with around 50% of fragment remaining after 7 hours. In contrast, in WKO DCs only 20% of the FL and 10% of cleaved TLR9 protein was degraded after 7hrs of chase (*Fig 4.23B*), indicating a significantly slower degradation rate. After 16 hours of chase, the last time points analysed all receptor was degraded.

This experiment clearly demonstrates that impaired degradation of cleaved TLR9 leads to a longer persistence of the signalling receptor in WKO cells.

4.6 Trafficking and Degradation of TLR9 ligands

4.6.1 Analysis of trafficking of exogenous TLR9 ligand

We next assessed the trafficking of TLR9 ligands in WT and WKO DCs. We first examined the fate of exogenous ligand. We fed the cells with labelled CpG-B (CpG-B/FITC) and analysed the cells by confocal microscopy at different time-points after pulsing (10, 20, and 60 minutes) (*Fig 4.24A*). At 10 minutes we observed several CpG-B structures scatter in the cytoplasm. By quantifying the area of CpG-B containing vesicles we found that intracellular CpG-B clusters had a significantly larger size in WKO than in WT (*Fig 4.24 B*). After 60 min very few WT cells contained intracellular CpG-B/FITC, whereas CpG-B positives structure were still detected in the cytosol of 20% of WKO cells (*Fig 4.24 C*). CpG-B overlapped with early endosomes at 10 minutes but colocalization was lost after 20 minutes, indicating translocation to a later compartment. On the contrary, in WKO DCs the overlap between CpG-B and early endosomes was present as well after 20 minutes at early and latest time point (*Fig 4.24D*).
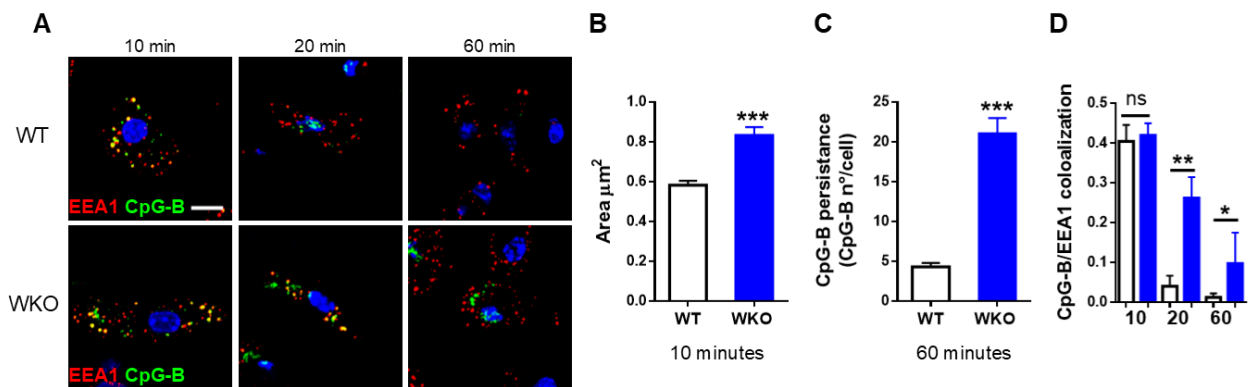
Figure 4.24 Large intracellular aggregates of CpG-B in WKO DCs with impairment in degradation. $2x10^5$ WT or WKO DCs were incubated with CpG-B/FITC at 37C for different time point, as indicated. Cells were seed on coverslips and labelled with EEA1 marker. (**A**) Images show one representative confocal plane of WT and WKO DCs scale bar 10 µm. (**B**) Graph show the volumes of intracellular CpG-B/FITC at 10 minutes, (**C**) CpG-B/FITC persistence at 60 minutes and (**D**) Mander's overlap coefficient (MOC) for colocalization between EEA1 and CpG-B/FITC at different time point. Quantification on 20 cells/genotype. All the data in the figure are means ± SEM *p≤0.05, **p≤0.01 ***p≤0.001

4.6.1 Analysis of trafficking of endogenous TLR9 ligand

To reinforce the relevance of this observation in the context of autoimmunity we next examined the fate of endogenous TLR9 ligand, ie., using the IC complexes that were presented in section 4.1.2. For this experiment cells were pulsed for 7 minutes with 90 ng/ml of IC, washed and chased for the different time points. To analyse the intracellular localization of IC, we used antibodies to IC and EEA1 for the early time point, 7 and 15 minutes, and LAMP1 for the late time point, 40 minutes. We found that WT DCs showed a complete colocalization between IC and early endosomes 7 minutes after internalization and that the overlap decreased after 15 minutes. After 40 minutes all ICs within WT cells merged to LAMP1, indicating that in WT cells IC have reached degradative organelles. Progression of IC was delayed in WKO DCs, as Mander's overlap coefficient with EEA1 was still high after 15 minutes (MOC 0.7), indicating that ligand persist in longer in early compartments. After 40 minutes of incubation we still detected large IC structures not fully merged to LAMP-1 (*Fig 4.25A*). Analysis of the size of IC aggregates at 15 and 40 minutes showed that, like exogenous CpG-B, WKO DCs accumulates cargo in larger vesicles as compared to WT DCs (*Fig 4.25B*).
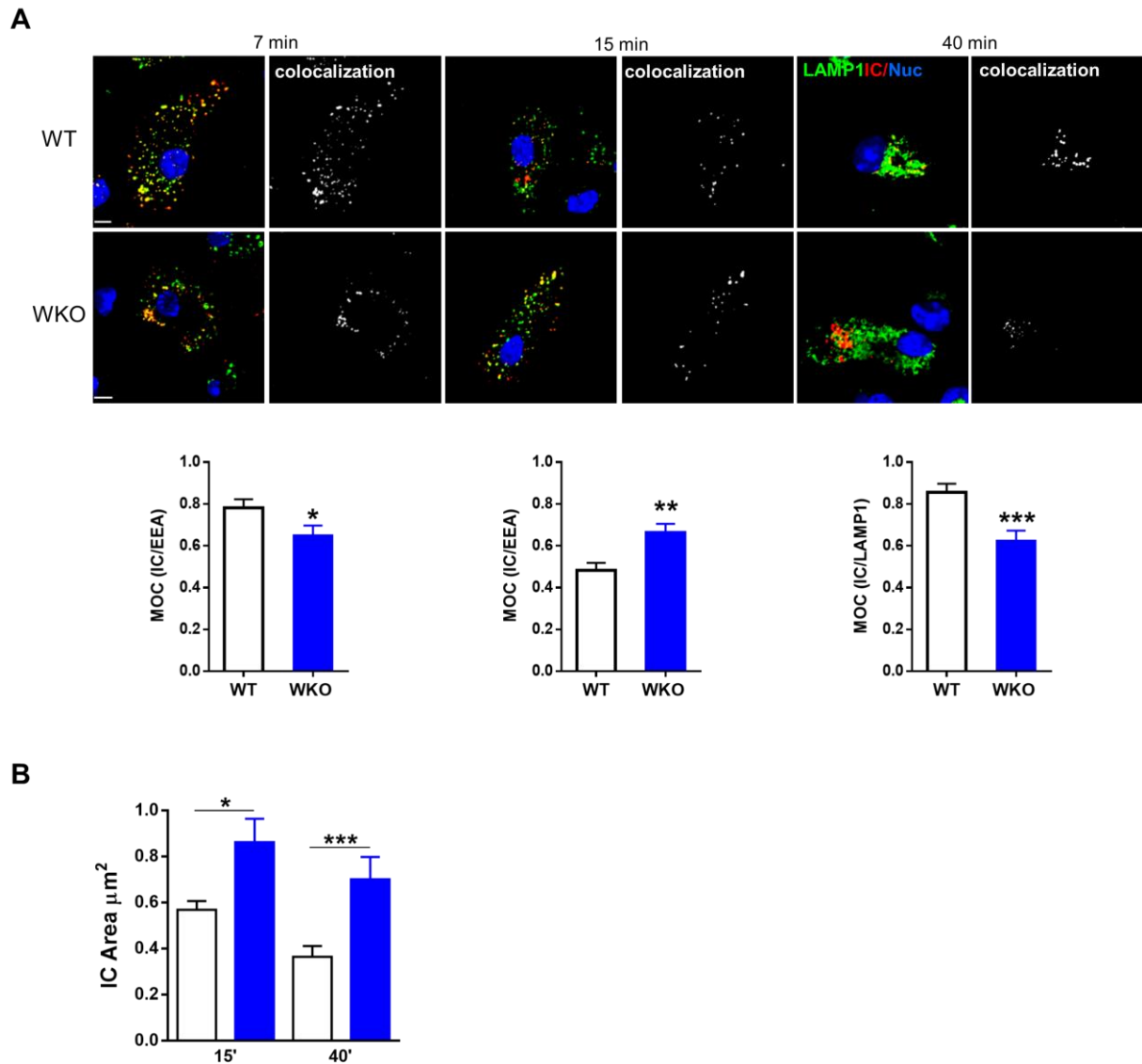
Figure 4.25 Large intracellular aggregates of IC in WKO DCs with impairment in degradation. (**A**) WT or WKO DCs were pulsed with 90 ng/ml IC for 7 min and chased for different time points, as indicated. Cells were labelled with EEA1 or LAMP1 markers. IC was detected using 594/Fab. Images show one representative confocal plane, together with the corresponding co-localization mask for EE or LAMP1 and IC of WT and WKO DCs. Scale bar 5 µm. Graph shows the relative Mander's overlap coefficient (MOC) between IC and EEA1 or IC and LAMP1. Quantifications on 20 cells/genotype. (**B**) Analysis of total IC area in WT and WKO DCs at 15 or 40 minutes. All data in the figure are means ± SEM *p≤0.05, **p≤0.01 ***p≤0.001.

Based on this analysis we conclude that translocation of internalized IC across the endocytic pathway and delivery to lysosomes is delayed in WKO cells. To confirm this observation, we followed degradation of IC by Western Blot. Cells were pulsed with IC, washed and collected after 20, 45 and 90 minutes to prepare whole lysates. As shown in *Figure 4.26* the amount of intracellular IC decreased after 45 and 90 minutes of incubation in WT cells indicating degradation. In WKO DCs the amount of IC remained equal at 20 and 45 minutes and only decreased after 90 minutes.

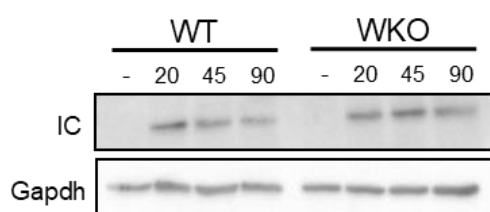**Figure 4.26 Delay of IC degradation in WKO DCs.** (**A**) Representative western blot of IC degradation in cells pulsed with 20 ng/ml. 3×10^5 were lysate and detected with an anti-IC antibody and anti-Gapdh antibody as loading control.

In sum, these data indicate that WASp is necessary to orchestrate trafficking of endogenous TLR9 triggers across endo-lysosomes and to target them for degradation.

4.7 Enhanced TLR9 proximal signaling in WKO DCs

4.7.1 Increased activation of TLR9 signalling pathway in WKO DCs

Thus far our data showed that TLR9 triggering by exogenous and endogenous ligands induces increased production of type-I interferon in cells lacking WASp and that this may depend on accumulation and persistence of ligands within "maturation-defective" endocytic compartments. In this section we addressed the early signalling events triggers by ligation of TLR9 in WT and WKO DCs.

Upon ligand recognition, TLR9 starts a signalling cascade that ultimately leads to cell activation and induction of inflammatory genes. Myd88 is the first adaptor recruited by the toll-Interleukin-1 receptor (TIR) homology domain at the C terminus of TLR9. Myd88 recruits and activate IRAK 1,4 and the IKK kinase complex (IKKα/β), that in turn activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activation and nuclear translocation of interferon regulatory factors (IRFs).

We here examined this cascade upon stimulation with IC in WT and WKO DCs. Cells were stimulated with the lowest doses of IC (10 ng/ml) for different time points (5, 15 and 45 minutes), lysed and analysed by Western blot. A faint phosphorylation of IKK complex was observed at later time points in controls whereas activation was clear as early as 5 minutes after IC stimulation in WKO DCs, in line with a lower threshold of activation. Similar to that, mitogen-activated protein kinase (MAP kinases) ERK1/2 is consistently phosphorylated only in WKO DCs after 45 minutes and hardly visible in WT cells. Downstream of ERK1/2, p-NF-κBp65 levels were higher in WKO than in WT at all time points (*Fig 4.27*).
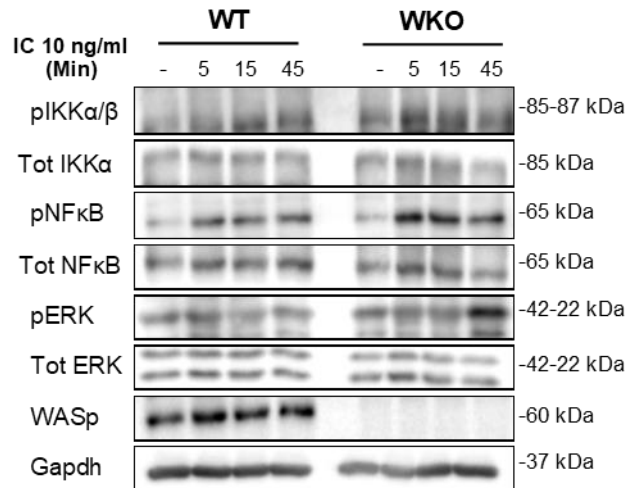
Figure 4.27 WASp deficiency leads to increased activation of TLR9 signaling pathway. $2x10^5$DCs were stimulated with IC 10 ng/ml for different time point, as indicated. Whole cell lysates were analysed by western-blotting for IKKα/β, p-IKKα/β, NF-κB, p-NF-κB, ERK p-ERK, WASp and Gapdh. Representative images are shown from three independent experiments

4.7.2 Analysis of IRF1 nuclear translocation

As mentioned before, MyD88 is a central adaptor in the TLR9 signalling cascade. A recent study demonstrated that induction of type-I interferon in DCs is mediated by IRF1 that interacts with MyD88 upon TLR9 activation. Upon binding to MyD88-associated IRF1 migrates into the nucleus more efficiently than non-MyD88-associated IRF1 and participates in the TLR-dependent gene induction of IFNβ (Negishi *et al.*, 2006)

To investigate nuclear translocation of IRF1 in our model we used two approaches. First, we performed biochemical analysis to investigate IRF1 nuclear translocation. After IC stimulation cell lysate were fractionated into a nuclear and a cytoplasmic fraction and the nuclear fraction were analysed by western blot using antibodies to IRF-1 and Poly [ADP-ribose] polymerase 1 (PARP-1) as loading control for the nuclear fraction and tubulin as a negative control, to assess purity of our extracts. As shown in *Figure 4.28A,* at 45 minutes after stimulation the amount of nuclear IRF-1 increase of 1,2 folds in WT cells. The increase was much larger (1,8 folds) in WASp null cells indicating enhanced activation of the pathway downstream TLR9 and Myd88.As a parallel approach we analysed IRF1 nuclear translocation by immunofluorescence under the same conditions *(Figure 4.28B).* Control or IC stimulated cells were stained with anti-IRF1 antibody. In resting cells, the basal level of nuclear IRF1 is similar between WT and WKO DCs. In WT cells the index of colocalization of IRF-1 in the

nucleus between resting and stimulated cells did not differ substantially. In contrast we observed a significant increase in IRF1 nuclear translocation in WKO cells (2,2 folds ± 0,2 SD). As IC doses used to stimulate cells were very low (20 ng/ml), all these data suggest that WKO DCs have a higher activation threshold for endosomal TLR9 response.
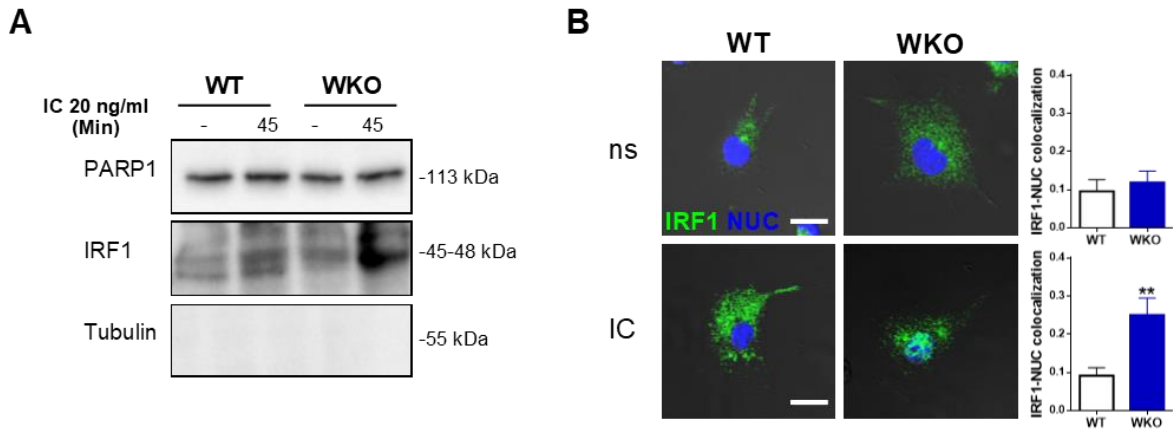
Figure 4.28 IRF1 translocate into nucleus in WKO DCs after IC stimulation. (**A**) Nuclear fraction cell lysates from unstimulated (-) or stimulated (45) DCs were analysed by western-blotting to detect IRF1 translocation, PARP1antibody is used as loading control and αtubulin as negative control. Representative images are shown from three independent experiments (**B**) WT or WKO DCs unstimulated or treated with 20 ng/ml IC for 45 minutes are stained with IRF1 antibody. Images show one representative confocal plane of WT and WKO DCs scale bar 10 μm. Graph show the relative Mander's overlap coefficient mean ± SEM between IRF1 and Nucleus. Quantification on 25 WT and 22 WKO **p≤0.01.

4.8 Actin perturbation mimics the phenotype of WKO cells

4.8.1 WASp is the main NPFs expressed in DCs

As mentioned before WASp belongs to the WASP/WAVE family of NPFs, and its expression is restricted to the hematopoietic lineage. We speculated that the phenotype observed in WKO DCs may be due compensation by other NPFs that become upregulated. To investigate this possibility, we examined mRNA expression levels by real-time PCR of four different NPFs in WT and WKO resting DCs (*Fig4.29*). In WT DCs WASp is highly expressed, whereas production of the other NPFs is very low. Moreover, in WKO cells no up-regulation of other NPFs is observed as the levels of WASH, N-WASp and WHAMM are equal to WT cells. In MEF cells used as control WASp expression is absent, and the other NPFs such as WASH and N-WASp are expressed at high level if compared to DCs.
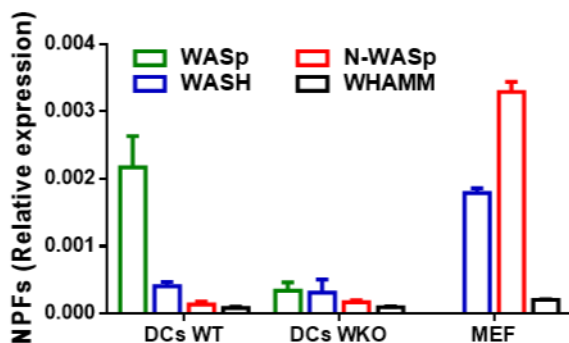
Figure 4.29 NPFs expression in WT and WKO DCs. Gene transcript in resting cells of WASp, WASH, N-WASp and WHAMM in WT, WKO DCs and MEF. Transcriptional levels were evaluated by RT-PCR, values are means ± SEM of two independent experiments.

These results strongly suggest that WASp is the main (exclusive?) activator of the Arp2/3 complex in DCs. This raises the interesting possibility that WASp may take the work of the other NPFs in the non-hematopoietic cells, such as intracellular trafficking, Golgi-ER transport, endosomal actin nucleation etc (see intro chapter 1.3.2). Accordingly, we have found a strong localization of WASp with endosomes and with Golgi (*Fig 4.12*).

4.8.2 Analysis of endosome structure in cells treated with actin inhibitors

We thus asked whether the phenotype of WKO cells is directly linked to its function as an activator of the Arp2/3 complex. To this aim we treated WT DCs with two actin inhibitors. CK666 is a specific Arp2/3 inhibitor that acts blocking the switch of the Arp2/3 from an inactive to an active conformation; Latrunculin A (LAT A), which sequesters G-actin monomers and prevents it from polymerizing. WT DCs were treated for 30 minutes before slide attachment with CK666 25µM and Latrunculin A 0,5 µM. DMSO was used as control and cells were stained to visualize early endosome (Fig. *4.30A*). We found that cells treated with DMSO contained several well-defined rounded vesicles with an average area of 0,5 μm^2 like WT DCs. In contrast cells treated with CK666 contain larger vesicle (0,7 μm^2) suggesting cluster of several fused endosomes like in WKO DCs. Furthermore, cells treated with the strongest inhibitor LAT A, presented collapsed vesicles with an average area of 1 μm^2, 2-fold higher than control cells (*Fig. 4.30B*).
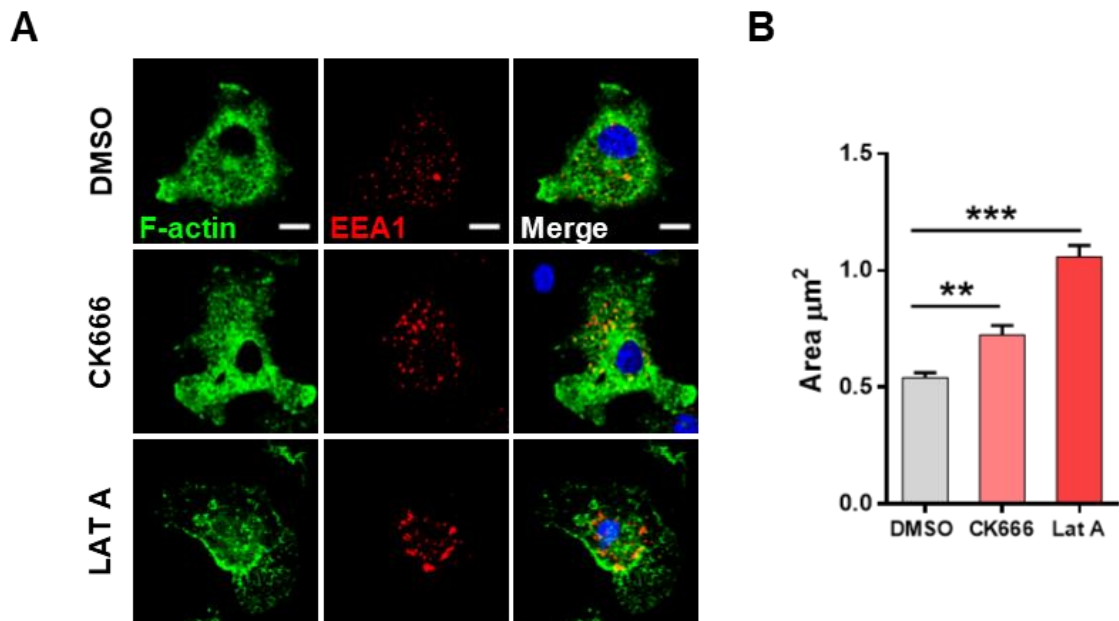
Figure 4.30 Enlarged endosome in cells treated with actin inhibitors. WT DCs were treated with two different actin inhibitors, CK666 25µM, LAT A 0,5 µM and DMSO as control for 30 minutes. (**A**) 1×10^5 cells were stained with antibody against EEA1 and phalloidin (F-actin). Images show representative single confocal planes with scale bar 10 µm. (**B**) Endosomes area was quantified on individual cells, data were collected from 20 cells for conditions, bars are endosome area means ± SEM, $p < 0.01$**, $p < 0.001$***.

From these results we can conclude that Arp2/3 regulates the morphology of endosomes and that its inhibition mimics the phenotype of WKO cells.

4.8.3 Analysis of TLR9 degradation in cells treated with actin inhibitors

From previous results we concluded that inhibiting actin polymerization mimics lack of WASp in DCs, forming aberrant endosome structure. We next examined whether the treatment interferes as well with endosomal function and we evaluated TLR9 degradation. TLR9-HA expressing WT and WKO Hoxb8-DCs were pre-treated with actin inhibitors or DMSO for 30 minutes, washed and lysed immediately or incubated for three hours in the presence of 5 μM of CHX. Levels of FL and cleaved TLR9 were assessed by western blot (*Fig 4.31A*). We found that both inhibitors had an important impact in TLR9 degradation. In control cells, cleaved TLR9 receptor decreased of more than 40% after 3 hours in CHX. In contrast in cells treated with actin inhibitors the rate of degradation was reduced, with only 20% and 10% of degradation of the cleaved receptor for CK666 and LAT-A treated cells, respectively (*Fig 4.31B*).

This result demonstrates, that upon inhibition of actin polymerization TLR9 degradation is impaired and the active receptor persists longer.
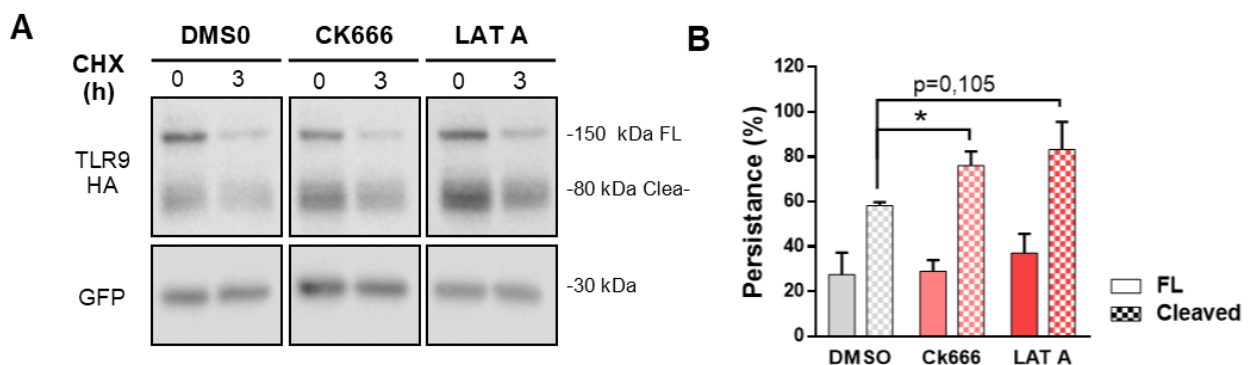
Figure 4.31 Reduction in TLR9 protein degradation in cells treated with actin inhibitors. (**A**) Representative western blot of TLR9 degradation in cells treated with CK666 25µM, LAT A 0,5 µM and DMSO as control for 30 minutes to inhibit actin polymerization and with 5µM CHX to block protein synthesis. The whole cell lysates were detected with an anti-HA and anti-GFP. (**B)** Bars show mean ± SEM band persistence (%) normalized to GFP with time 0 of three independent experiment *p≤0.05.

Ongoing experiments will define the rate of degradation of TLR9 ligands in cell treated with actin inhibitors.

4.8.4 Enhanced signaling in cells treated with actin inhibitors

IRF1 nuclear translocation showed to be an easy and reliable method to assess TLR9 activation and signalling in our model. We decided to investigate IRF1 nuclear translocation after IC stimulation by immunofluorescence in cells treated with actin inhibitors. We pre-treated cells with inhibitors followed by stimulation with IC for 45 minutes (*Fig 4.32A*). Single cell imaging and quantification of the distribution of IRF1 with respect to the nucleus showed a significantly higher nuclear localization in cells treated with CK666 with respect to DMSO treated cells (*Fig 4.32B*). Cells treated with LAT A showed a deeply altered cell morphology and displayed a little, but not significant, increase in IRF1 nuclear translocation, possibly because of toxicity of the treatment.
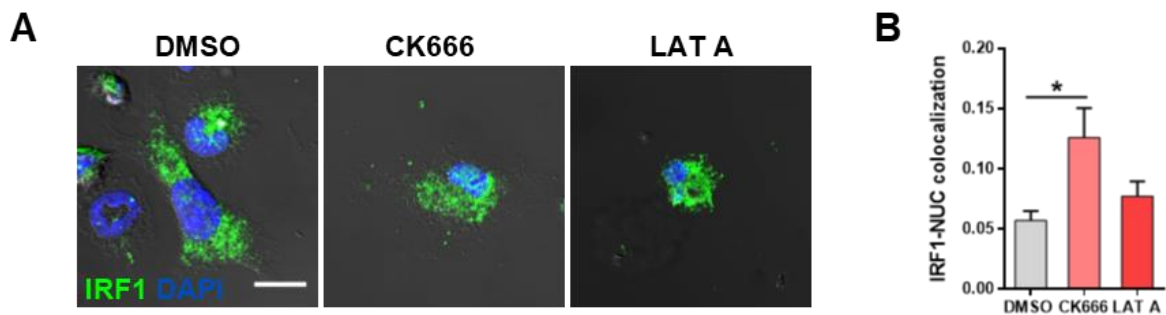
Figure 4.32 Increase in IRF1 nuclear translocation in cells treated with actin inhibitors. (A) Images show one representative confocal plane of cells treated with actin inhibitors, stimulated with 20 ng/ml IC for 45 minutes and stained with IRF1 antibody. Scale bar 10 µm. (B) Graph show the relative Mander's colocalization coefficient mean ± SEM between IRF1 and Nucleus. Quantification on 34 DMSO, 29 CK666 and 28 LAT A, *p≤0.05.

This result demonstrates that in cells treated with CK666, IRF1 translocate into nucleus after IC stimulation, suggesting that these cells have a lower threshold for TLR9 activation.

To directly address this hypothesis, we tested the functional responses of DCs treated with actin inhibitors using IC. We used the maximal IC dose that induces a response in WKO cells but not in WT cells (refer to *Fig 4.2B*). Transcriptional activation of the IFN-β gene was absent in control cells whereas treatment with the inhibitor of Arp2/3 for 30 min before IC

addition strongly activated gene transcription (*Fig 4.33*). Treatment with LAT-A, despite a possible toxic effect, was still capable to render cells responsive to IC stimulation.
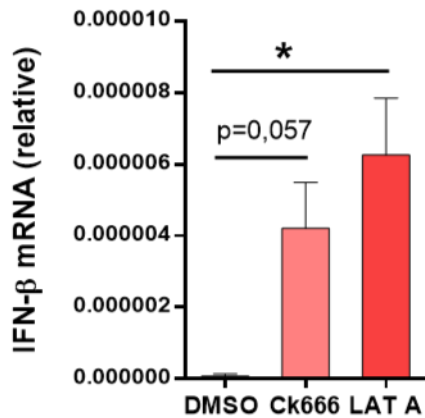
Figure 4.33 IC stimulation induces IFN-β secretion in cells treated with actin inhibitors. 3×10^5 DCs were stimulated 90 ng/ml of IC IFN-β gene transcript was measured after 3 hours of stimulation. Values are means ± SEM of three independent experiments. *$p \leq 0.05$.

These results demonstrate that controlled actin polymerization modulates responsiveness of TLR9 to incoming triggers and it strongly suggest that WASp acts as a negative regulator of TLR9 signalling by maintaining the proper structural and functional integrity of the endo-lysosomal system.

5. Discussion

Toll-like receptors (TLRs) are innate immune receptors, which play a key role in both innate and adaptive immune responses. TLRs trigger inflammatory responses by various biological mechanisms such as recruitment of inflammatory cells, cytokine production, or activation of adaptive immunity. Although the innate immune system is designed to recognize specific molecular patterns associated to different infectious pathogens, TLRs also detect several self-proteins and endogenous nucleic acids. Data rising from animal models and human patients indicate that excessive activation of TLRs signalling pathways by exogenous or endogenous ligands may carry to unwarranted inflammation with dangerous outcomes, including autoimmune diseases (Hosseini *et al.*, 2015). Subcellular compartmentalization of TLRs is essential to regulate initiation and termination of signalling avoiding excessive inflammation (Lee and Barton, 2014). Studies of mechanisms and adaptor that limit unrestrained activation of innate immune cells upon triggering of TLR are, therefore, of increasing interest as their potential in understanding autoimmunity.

In that context, the main goal of this work was to dissect molecular mechanism that lead to excessive production of type-I interferon in the context of Wiskott-Aldrich syndrome (WAS). We identified WAS-protein (WASp) as negative regulator of TLR9 signalling in DCs. In the last part of this manuscript the results presented will be discussed according to four axes that I wold like to highlight:

1) Development of new tool to study TLR9 responses in WKO DCs;

2) The importance of maturation in endocytic trafficking to control TLR9 signalling;

3) Dysregulated trafficking of TLR9 and its ligands in WASp KO DCs can explain excessive receptor activation;

4) Actin nucleation by WASp is important to negative regulate TLR9 signalling.

Development of new tool to study enhance TLR9 response in WKO DCs

Previous studies in our group have shown that absence of WASp, an activator of the Arp2/3 complex, controls TLR9 signalling in plasmacytoid dendritic cells (pDCs). Lack of WASp leads to increased type-I interferon production in pDCs and elevated levels of circulating IFN-I in the mouse model of the disease and contributes to the development of autoimmunity (Prete *et al.*, 2013). Therefore, understanding the mechanism underling this phenomenon represents a crucial accomplishment. Since pDCs are extremely difficult to obtain in large numbers and to manipulate I developed and validated cellular tools to better figure out molecular mechanism by which WASp protein regulate signaling.

First, we tested the responsiveness of bone marrow-derived DCs (BM-DCs), and we observed that both, IFN-β and inflammatory cytokines induced by stimulation of exogenous (CpG-B) or endogenous (IC) TLR9 ligands, are enhanced in WKO BM-DCs. WASp null BM-DCs showed enhanced sensitivity to low doses of TLR9 ligands indicating a lower threshold for activation. Analysis of interferon stimulated genes (ISGs) in resting cells highlighted constitutive transcription of these genes in WKO BM-DCs. All these data prove an IFN-axis alteration also in WKO BM-DCs making it a suitable model to investigate the underlying mechanism.

Several fundamental questions on DCs function have been addressed using DCs differentiated from bone marrow precursors. However, to study biological mechanisms and processes, primary cells have inherent limitations such as the number, the cell heterogeneity and the little permissiveness of the cells to genetic manipulation. Cell lines are often used in place of primary cells and we decided to set in place a new method recently published in Häcker laboratory (Redecke *et al.*, 2013). We generate Hoxb8 mouse hematopoietic progenitor cell lines from WT and WKO mice, we tested their potential to differentiate in DCs. Cell lines offer several advantages, such as they are cost effective, provide a pure population easy to use, supply an unlimited material and bypass ethical concerns associated with the use of animal and human tissue. They are often used in research in place of primary cells. However, care must be taken when interpreting the results as cell lines do not always accurately replicate the primary cells. Therefore, we decided to examine Hoxb8-DCs features compared to BM-DCs and to validate the phenotype of WKO Hoxb8-DCs with respect to WKO BM-DCs. We reported, in WKO Hoxb8-DCs an increase in total cell area and

migratory defects as reported previously in primary DCs (Noronha *et al.*, 2004; Prete *et al.*, 2013). Most importantly, the phenotype of our interest, i.e., enhanced IFN-I production upon TLR9 stimulation and decreased threshold for activation, was conserved in WKO Hoxb8-DCs.

A major advantage, of the immortalized DC lines that I have generated during my work, has been the possibility to create a stable line of WASp null DCs starting from WT cells. Cells WASp$^-$ generated by CRISPR/CAS9 confirmed a significantly higher IFN-I production upon TLR9 stimulation as compared to WASp$^+$ control cell. This result is important as it definitely proves that hyper-responsiveness is not (or at least not only) a consequence of priming by the inflammatory environment from which cells arise and demonstrates that WASp controls TLR9 responses in a cell autonomous fashion. Previous data by siRNA gene knockdown in pDCs went in the same direction but with the limits associated to poor reproducibility of transient transfections in primary cells.

More generally, the Hoxb8-DCs line has the potential to extremely expand the range of experimental parameters, conditions and candidates to be considered by allowing extensive experimentation.

The importance of maturation in endocytic trafficking to control TLR9 signalling

Actin polymerization plays an important role in many membrane trafficking events including endocytic internalization and transport. One function of actin nucleation common to all these processes is the dynamic shaping and remodelling of membranes. The actin nucleators that contribute to these processes include the Arp2/3 complex and its Nucleator-promoting-Factors (NPFs) (Firat-Karalar and Welch, 2011). Among these NPFs, WASH has been found to localize on early and recycling endosomes, where it stimulates Arp2/3 activity and controls endosomal sorting, (Derivery *et al.*, 2009; Rotty, Wu and Bear, 2013). N-WASp promotes F-actin that would generate inward forces to detach vesicles from the plasma membrane in combination with the pin-chase activity of dynamin and it was shown to be essential for uptake of surface receptor like IL-2R (Grassart *et al.*, 2010; Molinie and Gautreau, 2016). WHAMM coordinates actin and microtubules networks and colocalizes with autophagy markers that emerge from the ER and develop into autophagosome (Campellone *et*

al., 2008; Kast *et al.*, 2015; Liu *et al.*, 2017). WASp is expressed exclusively in hematopoietic cells and lack of WASp in DCs is associated with abnormally F-actin distribution and fail to make sustained ruffles, lamellipodia, and filopodia (Calle *et al.*, 2004; Massaad, Ramesh and Geha, 2013). Moreover lack of WASp results in complete abrogation of podosomes in human, while in mice there is a disorganized cluster of F-actin dots resulting in a poorly ordered podosomal structures (Burns *et al.*, 2004; Calle *et al.*, 2004; Massaad, Ramesh and Geha, 2013; Moulding *et al.*, 2013)

Although a lot of studies are performed to understand the role of the different NPFs in non-hematopoietic cells, less is known about the relative expression and specific function of individual NPFs in hematopoietic cells and in particular on the presence of an extra hematopoietic specific NPF. This question is relevant as immune cells are highly specialized in actin-supported processes such as migration, internalization and endocytic trafficking pathways used for pathogen destruction and antigen processing. To begin addressing this question in DCs we have investigated the expression of WASH, N-WASp and WHAMM in WT. The analysis suggests that WASp is expressed more abundantly than any other factor in DCs with a slight expression of WASH and low to undetectable levels of N-WASp and WHAMM. Moreover, the analysis ruled out compensatory up-regulation of others NPFs in WKO DCs, as expression levels were similar to those in WT cells. A quantitative analysis of protein expression for N-WASp, WASH and WHAMM is still required to make a clear conclusion on the relative expression level. However, it is intriguing to speculate that WASp itself may be involved in regulating various processes accomplished by the different factors in non-hematopoietic cells. The reason for using a single instead of multiple NPFs may be related to the fast changes and adaptations in actin remodeling to which DCs are exposed to support the rate of migration/endocytosis, processes that recently emerged to be mutually regulated (Thiam *et al.*, 2016).

In support of the possibility that WASp may regulate more intracellular functions than previously known is its localization in proximity of early-endosomes and Golgi apparatus suggesting a role in endosomal nucleation and Golgi structure/function. Prompted by this finding we underwent a deep quantitative analysis of the morphology of the endocytic compartment in WT and WKO DCs and we observed multiple signs of alteration in WKO

DCs. We reported an important dysregulation in size from early to late compartments, including the Golgi apparatus. Early-endosomes structures are enlarged and collapsed suggesting a clustering of several fused endosomes, with lack of fission, all these data are in line with what reported in WASH-knockout cells (Gomez *et al.*, 2012). Moreover, our data underline alterations in morphology also at the Golgi level. WKO DCs stained with Rab6 marker display increase in size and also fragmentation. These results are consistent with what was shown for WHAMM. Campellone and colleague demonstrated that WHAMM interacts with Arp2/3 to promote actin assembly at the Golgi apparatus and along tubular membranes causing disruption in localization and organization of Golgi when overexpressed or silenced (Campellone *et al.*, 2008). While a defined role for WASp during vesicular transport requires further investigation, from these data we can suppose that WASp is required, at least in part, to control organelles fission and maturation.

Endosomes are sorting platform for receptors and cargoes taking different routes like recycling and degradation, and all these steps are mediated by cytoskeletal forces (Maxfield and McGraw, 2004). We reasoned that alterations in the structure of early endocytic compartments could be associated to a decrease in maturation of endosome and degradation pathway by late endosome and lysosome. Indeed, we have observed that Tf recycling was significantly impaired in WKO cells. This fits with a study reporting decreased Tf recycling in WASH depleted cells (Derivery *et al.*, 2009). Defects observed in Tf recycling could be the consequence of a defective fission of transport intermediates. Moreover, we tested acidification in intracellular compartment, with dextran pHrodo probe, since a proper vesicular pH balance and optimal acidification are critical for transporting and degrading cargo via the endocytic pathway and TLR9 cleavage as well. From our preliminary result, we found equal percentage of positive cells to pHrodo at initial steps, with an increasing delay in WKO DCs acidification at later time point. This experiment suggests that the pH in WASp null DCs decreases normally up to a point after which further acidification in prevented. A pH calibration curve with dextran pHrodo will help to define the exact pH values reached by organelles in WT and WKO cells.

A more definitive demonstration of defective endo-lysosomal maturation in WKO cells is provided by the DQ-BSA cargo degradation assay. Initial attempts using cell fixed at different

time points of chase after DQ-BSA intake have been difficult to control and yielded variable results. Finally, using the continuous acquisition mode in the flow cytometer to follow the kinetics of probe powering-on in real- time, we have been able to catch significant differences in the rate of fluorescence emission, revealing a slower degradation in WKO cells. Importantly, simultaneous loading with a pH/proteases insensitive probe (Alexa647-BSA) allowed to normalize for probe intake. It has to be noted that, contrary to what we could have expected from previous studies showing defective phagocytosis, uptake of dextran was not modified in WKO cells, over many experiments, leading to the conclusion that micropinocytosis in not controlled by WASp. Overall these data indicate that WASp controls cargo sorting at the levels of early endosomes and that further progression toward late endosomes and lysosomes is hampered. A link between defective sorting and maturation is in line with recent work in which was demonstrated that Neuropilin-2 (NRP2), a non-tyrosine kinase receptor frequently overexpressed in various malignancies, is a regulator of endocytosis. Lack of NRP2 leads to impaired endocytic transport of cell surface EGFR, arresting functionally active EGFR in endocytic vesicles that consequently led to aberrant ERK activation and cell death (Dutta *et al.*, 2017). This assay clearly showed that lack of WASp reduces DQ-BSA degradation rate without affecting the total amount of internalized BSA. Overall, these findings suggest that WASp is required for the intracellular trafficking of endocytic cargo from early-late endosomes to lysosomes for subsequent degradation, suggesting at least in part, that might facilitate cargo persistence inside cells that lead to continue signalling through the TLR9 pathway.

Dysregulated trafficking of TLR9 and its ligands can lead to autoimmunity

In the last years, the concept of TLR signalling being functionally intertwined with the cellular membrane trafficking machinery has received much attention. Tight control of active TLRs is critical for an adequate inflammatory response and is essential to prevent injury to the host (McGettrick and O'Neill, 2010; Lee and Barton, 2014). However, despite great progresses in the identification of the molecular components of TLR signalling pathways, still little is known about whether and how regulators of the endosomal trafficking system affect TLR signalling and function, particularly in autoimmune disease and under inflammatory conditions.

A remaining open question is what regulates TLR9-ligand translocation from signalling to degradative compartment. In this study we have demonstrated that WASp expression is important to control a fast rate of TLR9 degradation. Indeed, a similar delay in TLR receptor degradation was described in mutants for proteins that controls endo-lysosomal fusion such as Rab7b and Vps33B. It was shown that blocking Rab7b, endo-lysosomal trafficking is impaired causing a decrease in TLR9 and TLR4 degradation and an increased in signalling (Wang *et al.*, 2007; Yao *et al.*, 2009; Bruscia *et al.*, 2011). A recent study demonstrates that Vps33B, member of the HOPS complex (homotipic fusion and vacuole protein sorting) is critical for degrading activated TLRs and their ligands and its deletion leads to sustained presence of TLR4 in endosomes, and excessive inflammatory responses in macrophages (Akbar *et al.*, 2015). WASp may control endo-lysosomal fusion either providing the force for endosome fission and sorting (Liu *et al.*, 2006; Romer *et al.*, 2010) or by controlling docking and progression on microtubules. WASp has been shown to link the actin and microtubule cytoskeleton via Cdc42-interacting protein-4 CIP4, an F-BAR protein important for membrane curvature (Banerjee *et al.*, 2007).

Parallel to decreased degradation of TLR9 we found that exogenous (CpG-B) and endogenous (IC) TLR9 ligands engulfed by WKO cells traffic slowly across the first endocytic organelles and are degraded less efficiently than in WASp expressing cells. It emerges that WASp coordinates intracellular actin dynamics fast cargo translocation and delivery to degradative compartment. By doing so, WASp acts as a barrier against spurious activation ensuring that small amounts of endogenous ligand are rapidly disposed off.

Interestingly, a recent study reported that macrophages from lupus-prone mice have impaired lysosomal maturation, resulting in a decreased ability of lysosomes to degrade IgG-ICs and leakage of endosomal content into the cytosol (Monteith *et al.*, 2016). We hypothesize that prolonged persistence and defective progression to lysosomes could permeabilize the endo-lysosome membrane also in WASp leading to activation of cytosolic sensors. Further experiment will be performed to investigate the possible activation of cytosolic sensor by IC.

TLR9 signalling through the adaptor protein Myd88 activates two distinct pathways, one leading to the transcription of proinflammatory cytokine via NF-κB, and the second leading type-I-IFN through IRF1 nuclear translocation (Zheng *et al.*, 2009; Zhao, Jiang and Li, 2015). First, in agreement with previous observation (Abe *et al.*, 2009), we found that IC induce phosphorylation of the mitogen-activated protein kinase ERK but also complex IKKα/β and NF-κB as a consequence of TLR activation. Stimulation with low doses of IC induced a greater phosphorylation in WKO DCs than in WT DCs. As previously reported for autoimmune diseases IRF1nuclear translocation is higher in Sjögren syndrome patients (Zheng *et al.*, 2009) and SLE patients (Zhang *et al.*, 2015). Notably, we found IRF1 nuclear translocation only in WKO DCs, stimulated with low doses of IC. Together our results indicated that lack of WASp lead to hyperresponsiveness to endogenous TLR9 agonists that were shown to be retained into endosome during signalling (C. Honda, K. Ohba, Y. Hideyuki, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, 2005b).

Actin nucleation by WASp is important to negative regulate TLR9 signalling

Recent reports revealed the importance of cytoskeleton components in compartmentalized signalling during endocytic and phagocytic processes to start innate immunity and cellular self-defence (Mostowy and Shenoy, 2015). The role of actin polymerization is clearly crucial for uptake processes. However, roles for other cytoskeletal proteins in coordinating TLRs trafficking across the endosomal system are starting to emerge. It was reported that in pDCs, actin-dependent trafficking of TLR9 to lysosome-like organelles is essential to induce type-I IFN (Sasai, Linehan and Iwasaki, 2010) and here F-actin accumulation around TLR9 endosome is DOCK2 and RAC1 GEF dependent (Gotoh *et al.*, 2010). A recent study identified insulin-responsive aminopeptidase (IRAP) as major cellular compartments for the early steps of TLR9 activation in DCs. IRAP interacts with the actin-nucleation factor FHOD4 to control TLR9-ligand trafficking toward lysosomes Lack of IRAP leads to enhance DCs activation following by bacterial infection (Babdor *et al.*, 2017).

Further studies will be required to elucidate the detailed molecular mechanisms and the role of membrane-associated proteins, cytoskeletal elements and actin-binding proteins in endocytic and phagocytic processes.

Previous studies have reported the strict correlation between actin filaments in controlling early endosome formation, and that inhibition of actin dynamics led to the formation of enlarged endosome structure and impaired transport. Our imaging experiment reported that treatment with actin inhibitors (by different approaches) in WT DCs causes an increase in the size of the EEA1$^+$ vesicles, with appearance of irregular tubular endosomal structure, comparable with vesicles formed in WKO DCs. This scenario resembles what observed previously in cells depleted of cortactin, an interactor of WASp, and to reports where depolymerizing drugs were shown to induce block of early endosome segregation and inhibition of maturation/translocation of early endosome via the perinuclear region (Higgs and Peterson, 2005; Gauthier *et al.*, 2007; Ohashi *et al.*, 2011). During endosome maturation, the process of membrane remodelling, including tubulation, fission and segregation activities, is crucial as it sort internalized molecules to degradation, recycling to control the signalling fate. Interestingly, we found that inhibition of actin polymerization had an important impact in TLR9 degradation, suggesting a strictly regulated TLRs trafficking by actin nucleation. Importantly inhibition of Arp2/3 complex by CK666 treatment induced IRF1 nuclear translocation. Finally, and most compelling, we demonstrated that perturbing actin dynamics TLR9 signalling is enhanced leading higher production of type-I IFN, thus strengthening the notion that WASp restrains excessive activation via its actin regulatory properties.

In conclusion we have dissected the cellular mechanism of excessive TLR9 responses in WASp null cells. Using a controlled experimental system, we define a model whereby proper organization of endosomal actin nucleation mediated by WASp is required to maintain the threshold for TLR9 activation. Lack of WASp causes an aberrant endo-lysosomal structure, block recycling and sorting that in turn leads to accumulation of TLR9-ligand complex. This model has implications to understand WAS pathophysiology. In patients the inherent propensity of cells to overreact to small amount of ligand may synergise with multiple endogenous triggers leading to excessive inflammation and autoimmunity.

This study definitely places innate cells at the centre of the pathogenic loop that causes autoimmunity in WAS. Interestingly, a recent study demonstrates that WASp deficiency modulates autophagy and inflammasome function. They reported enhanced inflammasome activation monocytes from WAS patients and in WAS-knockout mouse dendritic cells using a model of sterile inflammation utilizing TLR4 ligation followed by ATP or nigericin

treatment, inflammasome (Lee *et al.*, 2017). The connection between the inflammasome and TLR9 are still poorly understood and it will be very interesting to address it in this disease model.

Finally, the implications of this study go beyond the pathophysiology of WAS, introducing actin as a new player in the complex scenario of the negative regulation of TLR signalling in DCs.
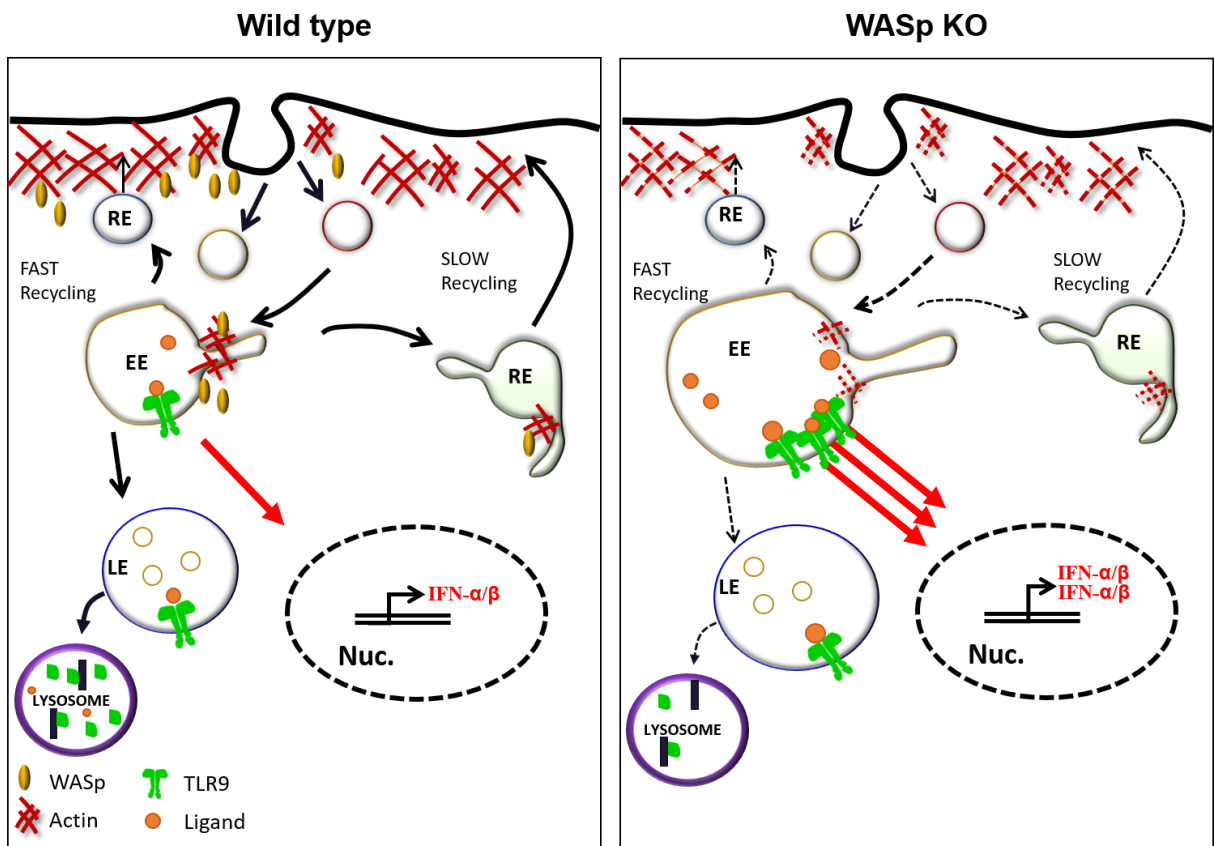
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