Scuola Normale Superiore

Pisa

The Wiskott-Aldrich syndrome protein in dendritic cells is required for polarization of the microtubules cytoskeleton, synapse formation and activation of T cells

Thesis submitted for the Degree of Doctor Philosophiae

Academic year 2008-2009

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ABSTRACT

The Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by recurrent infections, thrombocytopenia and a predisposition to develop autoimmune phenomena and lymphoreticular malignancies. The disease arises form mutations in the gene that codes for the WAS protein (WASp), a key regulator of actin dynamics in hematopoietic cells. Extensive analysis of WASp activation, regulation and function in T lymphocytes, have contributed to the understanding of the molecular basis of the immunodeficiency in WAS patients. However, it is increasingly evident that a general impairment of hematopoietic cell functions contributes to the pathogenesis of the disease

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) pivotal in the initiation of primary immune responses against pathogens and in the maintenance of peripheral T-cell tolerance against self-antigens. Despite clear indications of a role of WASp in the cytoarchitecture and migration of immature DCs, little is known about the effect of WASp deficiency on the ability of DCs to handle antigens and to interact with and activate naïve T-cells

The aim of this project was to extend the present knowledge on the biology of DCs by studying the role of WASp in several aspects of their activity and to increase our understanding of the role of DCs in WAS pathogenesis. Using a murine model of WASp deficiency (WASp-) we focused on four main topics in DCs. First, we evaluated the ability of WASp- DCs to internalize and process physiologically relevant forms of antigens. Second, we measured the ability to migrate and encounter naïve T cells *in vivo* and *in vitro.* Third, we studied the ability to physically interact, present antigens and form stable synapses with T cells. Finally, we evaluated the efficacy of WASp- DCs to support T-cell activation *in vivo* and *in vitro*. In this work we demonstrate that WASp is a key protein in DCs, required to properly activate T cells. We also reveal a new mechanism of polarization in DCs that supports the synaptic delivery of cytokines and enhances T-cell activation.

1. INTRODUCTION

1.1. Wasp Disease, general traits

The Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia, small platelet volume, eczema, recurrent bacterial and viral infections, autoimmune diseases, increased risk of malignancies and abnormal B- and T-cell function.

The disease was linked to a single gene composed of 1823 bp and 12 exons, which is present in the X chromosome and only transcribed in the hematopoietic cells. The product is a 502 amino acid called the Wiskott-Aldrich protein (WASp). The protein contains an N-terminal domain called EVH1 or WH1, a basic region followed by a 14 residue stretch (GTPase binding domain or cdc42/Rac-interacting binding motif) a proline-rich region containing multiple consensus motifs for Scr3 homology 3 (SH3) domain binding and an acidic C-terminal region (VCA domain).

The C-terminus of WASp is responsible of binding and activating the Arp2/3 complex, a potent nucleator of actin polymerization[1]. This domain is made up of a WASp homology 2 (WH2) sequence followed by a short central (C) sequence and a terminal acidic (A) sequence. WH2 domains bind monomeric actin [2], while the C and A sequences bind to the Arp2/3 complex.

The N-terminal domain binds to the WASp interacting protein (WIP) that regulates WASp
activity[3] . WASP also possesses a binding domain (CRIB, also called GBD) for the GTP-bound (activated) form of the small GTPase cdc42, as well as a basic sequence that binds to phosphatidylinositol 4,5-bisphosphate (PIP2) [4].

The native form of WASp suppresses its capacity to trigger Arp 2/3 complex-mediated actin nucleation. This autoinhibitory behavior is obtained by folding the GBD and the EVH1 domains to residues within the VCA domain. WASp activity is

evoked following cell stimulation by three different ways: disruption of the blockage by interactions between cdc42 and the GBD domain, interaction between SH3 domain-containing proteins and the proline-rich region, or by phosphorylation of tyrosine residues (Figure1).

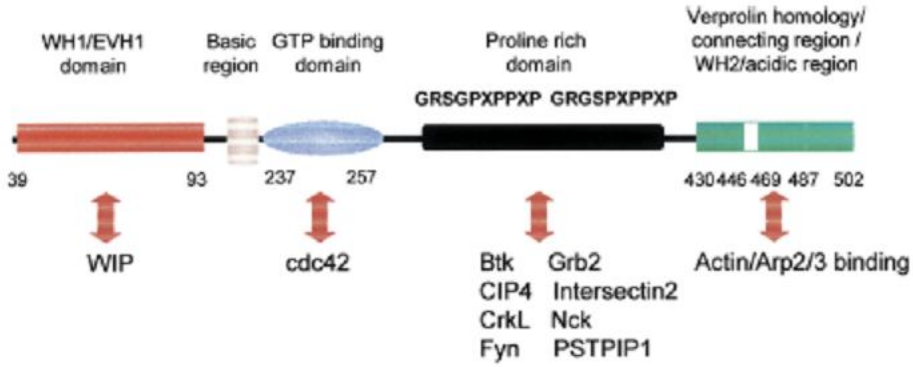


Figure 1. Functional domains of WASp and their interacting proteins. In the inactive state, WASP has an autoinhibitory conformation, with the molecule folded in such a way as to enable a stable interaction between the CRIB and C domains. This structural constraint is disrupted by interactions between activated cdc42 and the WASp GBD or, alternatively, between SH3 domain-containing proteins, such as PSTPIP1 and Nck, and the WASp proline rich domain.

WASp is a member of a family of proteins that participate in the transduction of signals from the cell surface to the actin cytoskeleton [5]. Other members of this proteins family are the three isoforms of SCAR/WAVE 1-3 and the ubiquitously expressed N-WASp.

The mechanisms responsible for the pathophysiology of WAS are directly linked to deficient actin organization in haematopoietic cells. The aberrant cell migration in hematopoietic stem lineage precursors has been proposed as the basis of the profound disorders of the immune cell development, functioning, and homeostasis in WAS patients[6]. However, the biological defects that have the most profound effects on the functioning of the immune system as a whole have not yet been clearly determined.

Actin remodeling represents an integral component of cell activation. Regulating the temporal and spatial distribution of actin polymerization is therefore essential for eliciting appropriate cell responses to environmental signals[7].

Several studies performed with lymphocytes from WAS patients have revealed both signaling and cytoskeletal defects, including aborted mitosis, abnormal pattern of actin filaments, impaired macrophage phagocytosis[8], NK cell cytotoxicity[9], reduced B-cell adhesion and migration[10] and T-cell activation[11 7]. The human WAS gene has 86% of similarity with the murine WAS gene. Thus, to better understand the role of WASp in the immune system, Snapper's group generated a WASp-deficient mouse containing a targeted disruption of the GBD/CRIB motif. This murine model share most of the pathology features reported in humans[5]. A summary of the cellular defects that have been reported[7] for the WASp-deficient mouse model is listed in Table 1.

Table 1. Functional defects in WASp-deficient cells

Cell type	Functional defects
Haematopoietic progenitors[12]	Decreased homing efficiency
Monocytes/macrophages[7; 13]	Abnormal morphology Absent podosomes Chemotactic/migratory defects Adhesion defects Phagocytic defects
T cells[11; 12 14]	Abnormal morphology Impaired CD3-mediated proliferation Impaired thymocyte development Impaired capping of actin and T-cell receptors
B cells[15]	Abnormal morphology with shortened microvilli
Platelets[16]	Reduced number and volume

Lack of WASp leads to defective development and maturation of T-cell and B-cell lineage cells in mice. However, it is not clear whether a functional redundancy of WASp and N-WASp is present. Cotta de Almeida et al. addressed this problem

generating a conditional double-knockout strain and suggested that T-cell development depends on the combined activity of WASp and N-WASp[17]. Nonetheless, WASp serves a unique role for peripheral T cell function. In peripheral T cells WASp plays a critical role in the reorganization of the actin cytoskeleton. WASp-deficient T cells show a reduced capacity to form an asymmetric patch of adhesion molecules and surface receptor following TCR engagement that leads to an impaired migration and proliferation[14; 16].

WASp plays a crucial role in the cytoskeletal regulation of B lymphocytes. WASp-deficient B cells are unable to respond to chemotactic gradients and have an impaired motility, caused by their inability to polarize the cell body[10]. Moreover, actin polymerization mediated by cdc42/Rac1, has been shown to be affected in WASp deficient B cells, causing an aberrant pattern of membrane protrusions upon stimulation with IL-4[15]. These defects have been proposed as an explanation to the aberrant peripheral B-cell maturation and homeostasis in WASp-deficient B cells (Westerberg 2008).

1.1.1. **Role of WASp in DCs**

Several defects in the cytoskeletal architecture of DCs such as aberrant podosome formation and altered membrane adjustments required for migration, have been reported[18] in human and mouse cells. To examine the functional role of WASp in DCs, most of the studies have been carried out using the murine deficient model. WASp- DCs fail to assembly podosomes during the response to chemotactic gradients, in particular, it has been shown that formation of podosomes and recruitment of the integrin Beta-2 is strongly compromised in the absence of WASp, causing a defect on the ability of adhesion to the Intracellular adhesion marker ICAM-1. ICAM-1 is a relevant molecule in stabilizing cell-cell interactions and facilitating endothelial migration. *In vitro* experiments resulted in abnormal DC membrane polarization and motility on a fibronectin matrix. Similarly de Noronha et al, shown *in vitro* impaired migration of WASp- DCs towards the chemokine

ligands CCL19, CCL3 and CCL21[19]. *In vivo*, WASp- DCs have a defective migration from skin and periphery towards the secondary lymph organs, which correlates with a reduced priming of naïve T cells[20].

1.2. Dendritic cells

DCs can be catalogued as a heterogeneous family of leukocytes that integrate information and transmit it to lymphocytes. Their main role is to recognize specific molecules upon infection of invading pathogens that trigger their differentiation into immunogenic Antigen Presenting Cells (APC), capable of priming and sustaining the expansion of naïve T cells.

Known as professional APC, DCs have a notable role in the initiation and modulation of the immune response due to their distribution and capacity to reach the lymphoid organs, areas where the CD4+ and CD8+ T cells accumulate. They have a unique capacity to translate the environmental signals into specific classes of adaptive immune responses by polarizing T-cell development. DCs have an inherent high efficiency for antigen presentation that allows them to induce strong T cell responses in small number and low levels of antigen[21]. DCs play also an essential role in the generation of both central and peripheral T-cell tolerance by inducing deletion, anergy or regulation of T lymphocytes[22].

1.2.1. **Subsets of dendritic cells**

The phenotypic and functional analyses of DCs found in thymus, spleen and lymph nodes have revealed that DCs are a heterogeneous population of cells that can be classified in several ways that are still debated. In general, they can be divided into 2 major populations: (1) non-lymphoid tissue migratory and lymphoid tissue–resident DCs and (2) plasmacytoid DCs (pDCs, also called natural interferon-producing cells)[21].

Migratory and tissue-resident DCs have two main functions: the maintenance of self-tolerance and the induction of specific immune responses against invading pathogens (figure 2)[23] , while the major function of pDCs is to secrete interferon-alpha in response to viral infections and to prime T cells against viral antigens.

1.2.1.1. **DCs in non-lymphoid tissues**

Non-lymphoid tissue DCs are present in the pancreas and the heart, at filtering sites such as the liver and the kidney, and at environmental interfaces as lung, gut, and skin. Among interface DCs, epidermal DCs also called Langerhans cells (LC) are the most studied. LC constitutively express major histocompatibility complex (MHC) class II and high levels of the lectin langerin[24]. These interstitial DCs express low levels of the integrin CD11b and coexpress alpha-Eβ7 (CD103), a ligand of the cell adhesion molecule E-cadherin expressed by most epithelial cells. In addition to CD103+ DCs, tissues also contain another major DC population that is characterized as MHC class II+ CD11c+ CD11bhi CD103–langerin.

Langerhans cells were first described by Steinman in 1978[25], who proposed a model where the DCs can be found in the epidermis or in the spleen. In this model, DCs can exist in two functional states: immature and mature. Immature DCs are located in the periphery and efficiently uptake self and non-self antigens but are quite inefficient for T cell activation. Only upon encounter of pathogens and mediators of inflammation, DCs enter to a development program called maturation. Maturation causes downregulation of their endocytic capacity, activation of the antigen processing machinery that generates complex of MHC molecules with peptides derived from the internalized antigens and an increase of T-cell costimulatory molecules. In parallel, changes in the pattern of chemokine receptors and adhesion molecules and modifications in the cytoskeleton structure induce the migration from the periphery to secondary lymphoid organs, where the antigens are efficiently presented to T cells, thus initiating adaptive immune responses.

In summary, tissue DCs uptake antigens and migrate continuously through afferent lymphatics vessels to the T-cell areas of lymph nodes (LN), a process that increases in response to inflammatory signals. The constant DC efflux from tissues to the tissue-draining LN requires constant replacement with new cells in order to keep the tissue-DC homeostasis[26].

1.2.1.2. **DCs in lymphoid tissues**

Lymphoid tissue–resident DCs are the most studied DC population in mice, but little information is available on their human counterparts. Lymph node DCs are a heterogeneous population as they include blood-derived lymphoid tissue–resident CD8α+, CD4+, double-negative spleen equivalent DCs, and migratory DCs entering via the afferent lymphatics that vary according to the LN draining site[26].

In mice, splenic DCs constitutively express MHC class II and the integrin CD11c. They are further classified into two major subsets that include CD4+CD8α–CD11b+ DCs that localize mostly in the marginal zone and CD8α+CD4–CD11b– DCs localized mostly in the T-cell zone[27]. CD4- CD8α- CD11b+ DCs have also been identified and are called double-negative DCs. CD8α+ DCs are specialized in MHC class I presentation, whereas CD4+ DC subset is specialized in MHC class II presentation. CD8α+ DCs have also been shown to cross-present cell-associated antigens, whereas CD4+ DCs are unable to do so[28]. The DC population present in the mucosa-associated lymphoid tissues, is equivalent to the one found in the spleen.

DCs that reside in the LN and in the spleen play a major role in the induction of immune response against blood-borne pathogens, since their function is to monitor the blood for the presence of infectious agents that spread through circulation. Intravenous inoculation of inflammatory compounds such as lipopolysaccharide (LPS) or CpG induce the activation of the LN DCs, their migration to the T-cell areas of spleen and LNs, the acquisition of a mature

phenotype and the secretion of large amount of IL-12, confirming their major role in immunosurveillance.

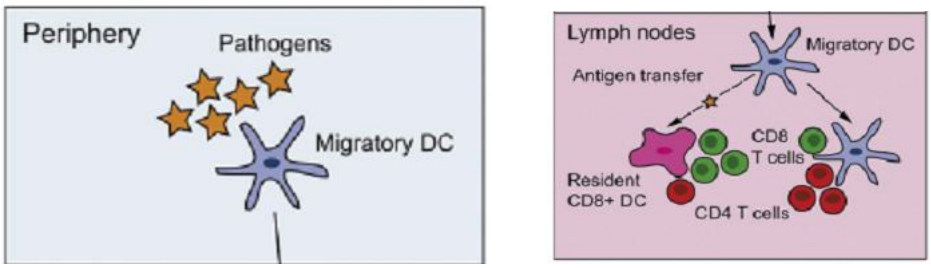


Figure 2. Role of the two main DC populations. Migratory DCs scan the periphery looking for pathogens, they drift away towards the lymphoid organs and encounter resident DCs, where they present those antigens to CD4 and CD8 T cells.

1.2.1.3. **Plasmacytoid DCs in lymphoid and non-lymphoid tissues**

Plasmacytoid DCs (pDCs) are a subset of DCs in both humans and mice with the ability to sense and respond to viral infections mainly by secreting large amounts of type I interferons. pDCs constitutively express MHC class II molecules and lack most lineage markers[29]. Murine pDCs lack CD11b and express low levels of the integrin CD11c and the lineage markers CD45RA/B220+ and ly6C /GR-1+, express PDCA1 and siglec-H, identified as a specific surface markers for mouse pDCs[30]. Human pDCs express very low to no level of CD11c, they express CD4 and CD45RA antigens, the c-type lectin receptor BDCA2, and the molecule BDCA4, a neuronal receptor often used to isolate pDCs, and high levels of the interleukin-3 receptor (CD123). pDCs constitutively express the IFN regulatory factor (IRF-7) that allows the rapid secretion of vast amounts of IFN-α in a signaling pathway that starts by engagement of Toll-like receptors 7 and 9[31]. pDCs circulate in blood and are found in steady-state at the spleen, thymus, LN, and the liver. Human and mice pDCs enter the LN through the high endothelial venules and accumulate in the paracortical T-cell rich areas. In contrast to the other DCs subtypes, pDCs do not efficiently migrate to peripheral tissue in the steady state with the exception of the liver[29].

1.2.1.4. ***In vitro* generated DCs**

The establishment of defined cell culture systems to generate DCs *in vitro* has been useful for functional and intracellular studies. The first method reported to differentiate mouse DCs *in vitro* involves cultures of bone-marrow or spleen precursors in medium that is supplemented with granulocyte/macrophage colony stimulating factor (GM-CSF), with or without interleukin-4 (IL-4). Surface marker examinations showed that the resulting DCs are partially similar to some of the lymphoid-organ-resident DC subsets found *in vivo*[32].

A more accurate system to generate lymphoid organ-like DCs emerged with the use of the FMS-like tyrosine kinase 3 ligand (FLT3L). Bone marrow precursors cultured with FLT3L differentiate into DCs with similar expression patterns of CD11b, CD24, CD172, Toll-like receptors, chemokine receptors and the ability to secrete IL-12 and the chemokine ligand 5 (CCL5)[33].

1.2.2. **DC specific functions**

The main function of DCs is the internalization of pathogens, followed by processing and presentation of antigen peptides to naïve T cells.

1.2.2.1. **Antigen uptake and pathogen recognition**

One of the properties that define DCs as potent antigen-presenting cells, is their ability to efficiently uptake particles and pathogens by endocytosis. Endocytosis is highly active in immature DCs and downregulated upon cell maturation, assuring the efficient sampling of the environment in the periphery and simultaneously limits the range of antigens that the cells will be able to present after leaving the marginal tissues[34]. There are three main endocytosis pathways: receptor mediated endocytosis, phagocytosis and macropinocytosis[35].

1.2.2.1.1. *Receptor mediated endocytosis*

The efficiency of endocytosis is increased by non-specific binding of solutes to the cell membrane and even more by the capture of soluble antigens through specific high affinity receptors that are concentrated into specialized endocytic transport vesicles. DCs express a wide range of endocytic receptors that are grouped into two main families. The first encloses receptors for the Fc portion of immunoglobulins (FcRs) and complement receptors (CRs) which are involved in the uptake of particles that are opsonized by immungloublins or complement factors. A second class of endocytic receptors comprises the scavenger receptors (SRs) and C-type lectin family receptors that directly recognize specific structures on both self-antigens and pathogens[36].

1.2.2.1.2. *Phagocytosis*

The uptake of large particulate antigens by phagocytosis is the prevalent form of antigen uptake *in vivo* for both pathogen-derived and endogenous antigens. The process starts with the engagement of specific cell surface receptors, that trigger a signaling cascade mediated by the Rho-family GTPases (Rho, Rac and Cdc42) that ends up with the extensive reorganization of the actin cytoskeleton, forming cell-surface extensions that zip up around the pathogen and engulf it[37].

The membrane protrusion and the activation of signaling pathways depend on the nature of the particle to be ingested and the receptors that recognize it. Phagocytosis might take place by engagement of specific receptors. In the FcR-mediated phagocytosis, the cells extend pseudopods that engulf the particle and subsequently fuse to form a phagosome, a process that requires the activation of cdc42 for the pseudopod extension and Rac for pseudopod fusion and phagosome closure. On the other hand, the CR-mediated phagocytosis does not induce pseudopod formation. The coordinated action of chemokines and integrin ligation controlled by Rho induces the formation of the phagocytic cup[38].

Phagocytosis by DCs is essential in host defense against infections. Immature DCs are able to phagocytose Gram positive and Gram negative bacteria, mycobacteria, yeast cells and parasites[39]. Additionally DCs have a main role in the clearance of apoptotic cells by the recognition of molecules that are absent on live cells, as calreticulin, phosphatidylserine and lysophospholipin.

1.2.2.1.3. *Macropinocytosis*

Macropinocytosis contributes to bulk fluid-phase uptake via the formation of membrane protrusions that collapse and fuse with the plasma membrane generating large endocytic vesicles that allow the sampling of large volumes of extracellular milieu. Macropinocytosis is constitutively active in immature DCs. This process is essential for the uptake of soluble antigens released by pathogens or externally provided upon intradermal or intravenous injection.

1.2.2.2. **Antigen presentation by DCs**

Upon internalization, antigens are degraded into small immunogenic epitopes that associate with the MHC and are transported to the plasma membrane where they trigger the activation of naïve T lymphocytes. In particular, the activation of CD8+ and CD4+ T lymphocytes requires recognition by the T cell receptor (TCR) of epitopes associated with MHC class I and MHC class II, respectively. The antigen processing pathways that lead to the formation of peptide-MHC complexes rely on proteolysis occurring in the proteasomes and lysosomes[35].

It is accepted that MHC class II molecules encounters exogenous antigens in the endocytic pathway and MHC class I is loaded with endogenous antigens in the endoplasmic reticulum (ER). However, it has been shown that MHC-II complexes can present intracellular antigens from intracellular proteins and MHC-I can present peptides derived from exogenous antigens, a process called cross-presentation[40; 41].

DCs perform antigen processing and presentation via MHC class I in proteins coming from alternative translation products, proteins found in the cytosol (either endogenous or viral) and proteins retrotranslocated to the cytosol and imported into the ER. Regardless the route of entry, most of the peptides loaded on the MHC-I complex are produced by the proteasome and further trimmed by cytosolic or ER resident peptidases. Proteins are trimmed in their N-terminal region by the proteasome and peptide products are shuttled in the ER where the final MHC-I peptide complex is formed and eventually presented on the cell surface[42].

DCs have the highest cross-presentation efficiency, allowing the entry of exogenous antigens into the MHC class I pathway of antigen presentation[43]. Soluble proteins, immune complexes, pathogens and cellular antigens have been reported to be cross-presented. However, the mechanisms by which the APCs transfer internalized antigens to the MHC class I loading pathway are not well understood. In most of the cases a limited endocytic degradation and transport into ER seem to be required, being a mechanism to allow the release of the antigen from the endocytic structures to the cytosol[44].

DC cross-presentation plays a key role in priming of CD8+ T cells in response to exogenous agents such as bacterial or viral infection, as well as in the maintenance of both, central and peripheral tolerance to self antigens by the deletion or anergy of self-reacting cytotoxic lymphocytes[45].

Antigens loaded on the MHC-II come from exogenous proteins that are internalized by DCs through different mechanisms of endocytosis or endogenous proteins that reside in the secretory system. The MHC-II is assembled in the ER and transported to early endosomes and further on to late endosomes and lysosomes, along this path it can bind polypeptide precursors that are trimmed by various proteases to end up reaching the plasma membrane[46] (Figure 3).

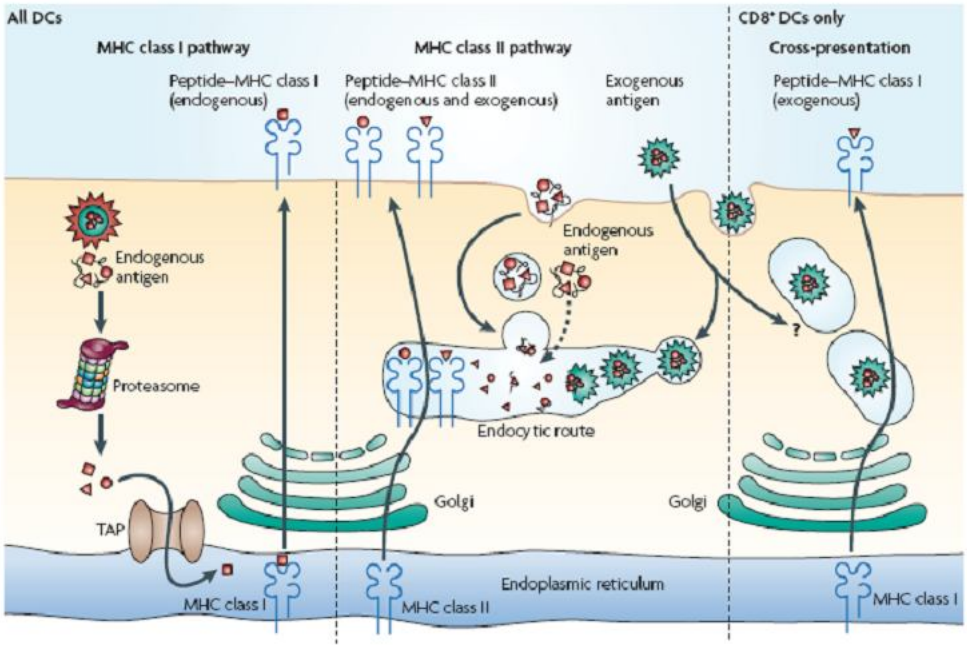


Figure 3. Antigen presentation pathways by DCs. All DCs have functional MHC class I and MHC class II presentation pathways. MHC class I molecules present peptides that are derived from proteins degraded mainly in the cytosol, which in most DC types comprise almost exclusively endogenous proteins. MHC class II molecules acquire peptide cargo that is generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment and also endogenous components. CD8+ DCs have a unique ability to deliver exogenous antigens to the MHC class I (cross-presentation) pathway, although the mechanisms involved in this pathway are still poorly understood. TAP, transporter associated with antigen processing[47].

1.2.2.2.1. *Dendritic cell maturation*

Concomitantly to the uptake and processing of the antigens, DCs recognize pathogen associated patterns (PAMPs) and sense inflammatory signals by different classes of membrane and intracellular receptors. Receptor engagement triggers signal cascades that lead to the production of inflammatory cytokines, upregulation of co-stimulatory molecules and to the altered expression of chemokine receptor profiles, providing them the ability to stimulate naïve T lymphocytes[48].

One of the best characterized classes of receptors that directly contribute to the inflammatory responses to pathogens is the Toll-like receptor family (TLR). Mammalian TLRs are a family of at least 12 transmembrane proteins that collectively recognize lipids, carbohydrate, peptide and nucleic acid molecules

expressed by different microorganisms, and differ from each other in ligand specificities, expression patterns and the inducible target genes[49].

Expression of TRLs 1, 2, 4, 5 and 6 is confined to the cell surface and appears to be specialized mainly in the recognition of bacterial products, where TLR4 plays an essential role in the recognition of LPS, a major component of Gram- bacteria. TLRs 3, 7, 8 and 9 are expressed on the membrane of endocytic vesicles or other intracellular organelles and are specialized in the detection of viral nucleic acids. This family of TLRs has an essential role in the antiviral immune responses mediated by the secretion of type I interferons. TLRs 9 and 7 are involved in the recognition of the 2'-deoxyribo(cytodine-phosphato-guanosine) (CpG) DNA motifs found in bacteria and viral DNA, while TLR3 is engaged by double-stranded RNA ('mimicked' by poly(I:C))[50].

Upon activation of their ligands, TLRs transduce signals through pathways involving diverse adaptor proteins containing Toll/IL-1R (TIR) domains. TIR activation triggers signaling cascades that end up with expression of host defense genes including inflammatory cytokines, type I interferon cytokines, the up-regulation of co-stimulatory molecules (CD40, CD80 and CD86), downregulation of chemokine receptors such as CCR1, CCR5 and CCR6 and upregulation of CCR2, 5, 7 and 19, which drives DCs into the afferent lymphatic vessels and the LN[51].

1.2.2.2.2. *DC trafficking*

After antigen uptake and processing, DCs sense and integrate different environmental signals and eventually migrate to T-cell rich areas of secondary lymphoid organs. There, antigen-specific immune responses are initiated by engagement of the TCR with the cognate peptide-MHC complex presented by the DC.

Antigen-loaded tissue DCs migrate to draining LNs through the lymph. These DCs penetrate the endocortex and reach the high endothelial venules (HEV). Thus, T and B cells that home to LNs by route of HEVs are first exposed to antigen loaded tissue DCs than lymphoid resident DCs. In this way, T cells are preferentially updated with information of antigens in the periphery[52].

The entry of DCs from peripheral tissues into the draining lymphatic vessels as well as their migration from the lymph into the LN cortex depends on the chemokine receptor CCR7 and its ligands CCL19 and CCL21. The prevailing model to explain how CCR7+ DCs arrive at peripheral lymphatic vessels is that they respond to a chemotactic gradient of CCR7 ligands, which originates from the lymphatic vessel. In mice, there are two known functional genes that encode CCL21. One form of CCL21, the CCL21-Leu, is expressed in the periphery by initial lymphatic vessels. The other form of CCL21, CCL21-Ser, is only expressed in the terminal lymphatic vessels and LN. The gradient caused by this divergent chemokine expression pattern might be the cause of DC migration[53].

DC migration from the periphery to the T-cell zone of lymphoid organs is a process that requires reorganization of the cytoskeleton and the plasma membrane. It has been shown that RAC1 and RAC2 are required for migration from the skin to the lymph nodes. Absence of both RAC1 and RAC2 strongly impairs the extension of dendrites by DCs and the mobilization of DCs to LN[54; 55].

Is widely accepted that migratory DCs have a major role in carrying outer antigens from peripheral tissues to the lymph nodes and these can be somehow presented

to the CD8+ DCs. Instead, self antigens are presented in the steady state on MHC class I and II molecules by both immature resident in lymphoid organs CD8+ DCs and mature migratory DCs, with the former being biased towards MHC class I cross-presentation and the latter towards MHC class II presentation.

Three different scenarios can be hypothesized for antigen-presenting functions of migratory and lymphoid resident DCs (Figure 6). A first situation is one in which the migratory DCs are themselves infected by a virus that has no deleterious effect on the DCs. In this situation, the migratory DCs are the main subset that presents endogenously produced antigens on MHC class I molecules [56]and MHC class II molecules, in the draining LN.

The second situation is one in which the migratory DCs are not infected, but the migratory DCs are still required to carry viral antigens to the lymph nodes, whether endogenously expressed or captured from infected cells, and transfer the antigens to the resident DCs in the form of endosomal vesicles or apoptotic bodies[57]. The main resident DC population that acquires the antigen would be the CD8+ DCs, due to their special ability to capture dead cells or cell fragments. The migratory DCs that survive and have not been inactivated by viral immunoevasins, as well as the resident CD8+ DCs, present the antigens according to their intrinsic abilities [58; 59].

The third scenario occurs in large scale infection of tissues that are drained by the lymph node. In this case the relative antigen presentation efficiency of the lymphoid and non-lymphoid DCs is almost the same on a per-cell basis, but the comparative number of migratory DCs that display antigen is much higher and their relative contribution to cross-presentation within the lymph node increases[59]. This hypothetical model implies the recruitment of monocytes to sites of inflammation, followed by their conversion into DCs. In this way, the monocyte-derived DCs may share some of the antigen-presenting functions that are initially carried out by the migratory and lymphoid-organ-resident DC subsets[60].

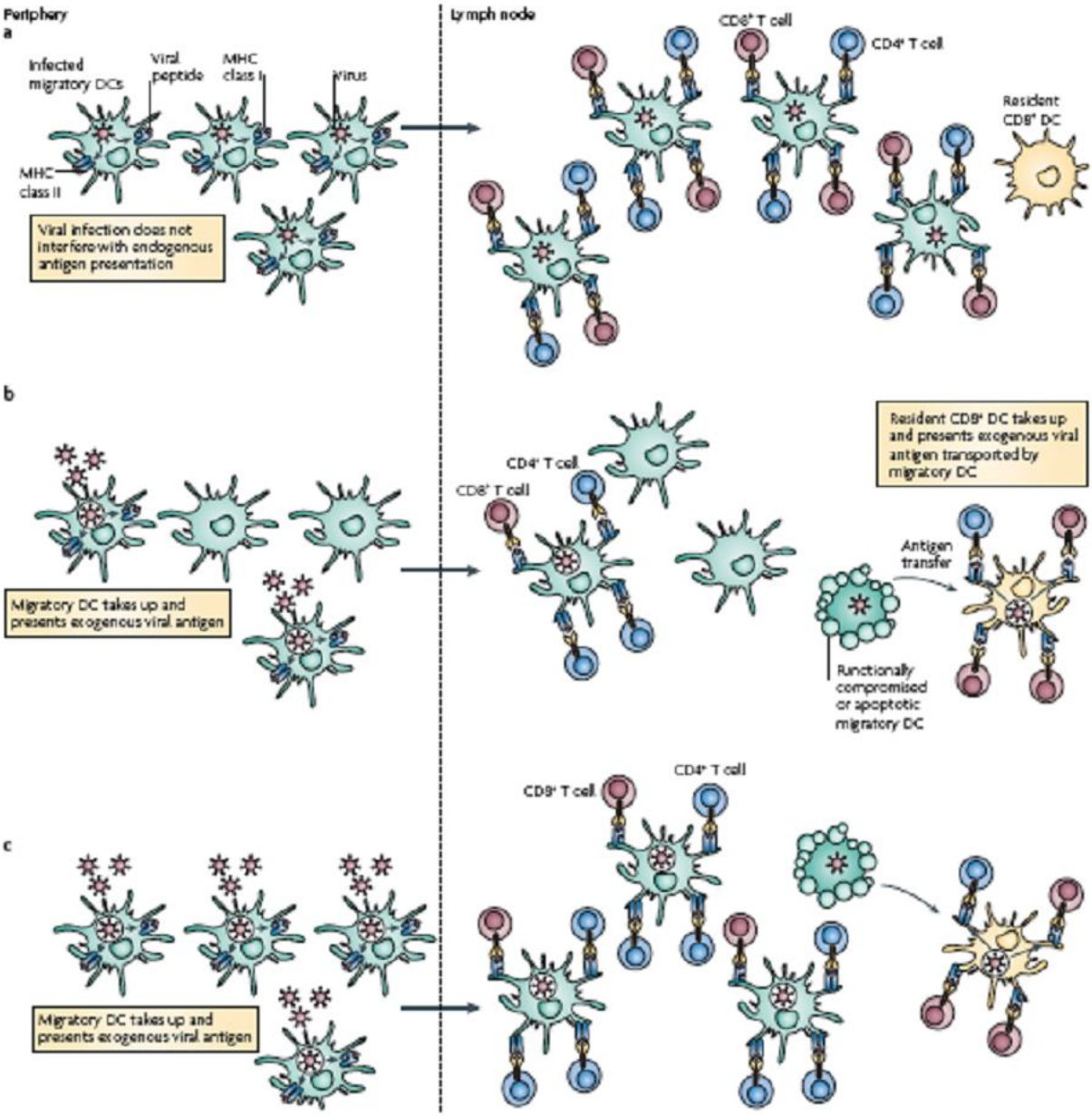


Figure 4. DC migration and antigen presentation. An scheme with three hypothetical scenarios showing how different populations of DCs may migrate and act in a synergetic way to counteract tissue infection[47].

1.2.3. **T-cell stimulation by DCs**

DCs play a crucial role in the induction of adaptive immunity and in the maintenance of peripheral tolerance to self and non-pathogenic environmental antigens. They can determine the fate of naïve T cells by three signals. The first occurs with engagement of the TCR with the cognate peptide-MHC complex, which triggers a TCR cascade that determine the antigen-specificity of the response. The second consists in the engagement of the CD28 and CTLA-4 co-stimulating receptors, to respectively influence the T cell response in a positively or negative way, controlling in this manner the initiation of the protective immunity. The last signal is delivered by cytokines and chemokines produced by DCs, which are sensed and integrated by the T cells. The combined effect of diverse released cytokines determine the proliferation, survival and ability to differentiate into effector cytotoxic T cells, Th1, Th2, Th17 or T regulatory cells[47].

It is widely accepted that DC maturation state is the critical switch that provides the signals for effector and memory T cell development, diverting T cells from anergy or deletion to protective immunity. On the other hand, immature DCs have been reported to have a inefficient antigen-presenting capacity, inducing peripheral T cell tolerance by antigen-specific T cell deletion, functional inactivation or by generation of regulatory T cells[61]. DCs capacity to efficiently prime naïve T cells resides in the increased expression of MHC complexes, adhesion and co-stimulatory molecules, which controls the stability and the duration of the DC-T cell contact[62]. In addition, mature DCs secrete enhancing cytokines like the interleukin-12 (IL-12) and interferons type I/II that support the survival and the differentiation into effector T cells.

1.2.3.1. **Immune synapse**

In order to deliver the three signals required to prime naïve T cells, a physical contact must be established with the antigen presenting cell (APC). The organized structure that takes place in the region of contact is called the immune synapse (IS). The formation of the IS is driven by different molecular mechanisms such as polarized recycling of receptors, passive lateral diffusion and cytoskeletal-mediated movement of molecules.

Immune synapses can have different morphological patterns with specific arrangements of membrane proteins and receptors. The simplest type of synapse experimentally observed has simple enrichment of receptors at the contact site. A more complex arrangement with central accumulation of both TCRs and adhesion molecules at the contact site is known as mature synapse[63].

The mature IS is composed of two concentric regions; first, the central supramolecular activation cluster (cSMAC), where the TCR, the co-stimulatory molecules (mainly CD40-CD40L and CD28-B7) and the intracellular signaling molecules PKCθ (105), lck, fyn, and ZAP-70 are concentrated in the inner side of the T cell membrane[63; 64]. Although it is accepted that the initiation of TCR signals occurs in peripheral microclusters that begin to form prior to IS formation, the cSMAC has been proposed as a place for TCR signal enhancement and TCR degradation[65] or as a site for enhanced receptor engagement and prolonged signaling [66]. Surrounding the cSMAC, a peripheral integrin-rich ring (pSMAC) is found, where LFA-1 interacts with ICAM-1 and which concentrates intracellular talin in the T cell provides an adhesive anchoring[64] (Figure 5).

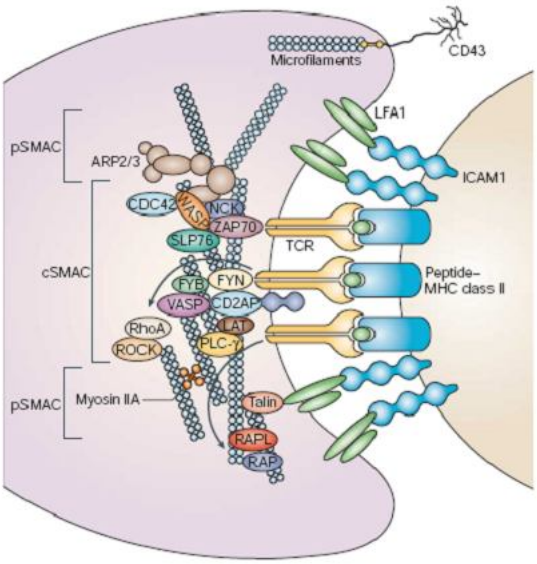


Figure 5. Structure of the Immune Synapse from the T cell side. TCR interaction with peptide–MHC class II molecules recruits tyrosine kinases such as ζ-chain-associated protein 70 kDa (ZAP70) and FYN, adaptor proteins and actin polymerization-regulatory molecules such as the small GTPases cdc42 and RhoA, WASP, inducing localized actin polymerization at the cSMAC through the ARP2/3 complex. The pSMAC is composed of leukocyte function-associated antigen 1 (LFA1) molecules that interact with ICAM1 expressed by the DC and also regulates the actin cytoskeleton through LFA1 interaction with talin[67].

1.2.3.1.1. *IS and T cell activation*

After formation of the IS, engagement of the TCR initiates a signaling cascade involving Lck, ZAP70, Itk and Vav, which result in the accumulation of Cdc42. Cdc42 in turns induces WASp activation and triggering of cytoskeletal rearrangements that results in polarization and activation of the T cell[67].

As the TCR is engaged, cortical actin concentrates at the contact region following its clearance towards the edges of the contact sites, in order to form a peripheral ring to the pSMAC[68]. These changes are thought to depend on increased membrane fluidity, caused by a transient dephosphorylation of ezrin, radixin and moesin (ERM), which blocks the crosslink of the actin cytoskeleton with the plasma membrane and provokes a decreased cellular motility. This in turn is associated with Ca^{2+} dependent phosphorylation and deactivation of the motor protein MyH9.

Accumulation of F-actin at the T cell-DC interface is the result of induced localized activation of multiple actin regulatory pathways, in particular the actin related proteins 2/3 (Arp2/3) complex. Activation of Arp2/3 is mediated by the interaction with WASp, WAVE2 and HS1. WASp is recruited to the site of TCR activation through its interaction with the SLP-76 associated adapter protein Nck, where it is activated via Vav-1 dependent stimulation of cdc42[69]. Vav-1 also activates Rac1, which results in the activation of WAVE2. However, experiments indicate that WASp deficient T cells still polymerize F-actin at the T cell–DC contact region, implying that WASp might control T cell activation in an alternative way, affecting the endocytosis and the exocytosis[70].

Inhibition of actin polymerization in T cells by cytochalasin D prevents synapse formation, and interferes with actin-myosin functions preventing movement of surface proteins to the contact zone[71; 72].

1.2.3.1.2. *T cell polarization*

Formation of the IS results in the polarization of the T cell, which is orchestrated by a protein network that includes four complexes, the Scribbled (Scrib), partitioning defect (PAR), Crumbs and a core planar cell polarity (PCP).

The Scrib complex consists of scrib, Lgl and Dlgh. In particular, Dlg has been found to be translocated to the IS, and it is thought to be responsible of reorganization of the cytoplasm, rearrangements of surface proteins and redirection in the transport of RNA and proteins[73]. Other proteins such as scrib, Crumbs 3 and Par3 rapidly relocalize to the IS, and their absence causes a reduction in the cell motility, conjugate formation and lytic activity[74].

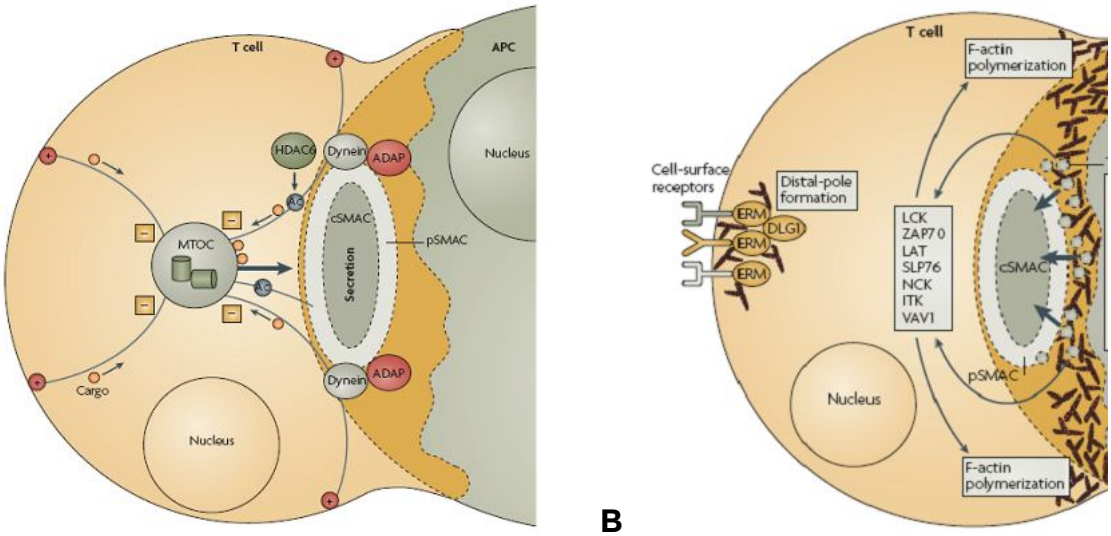
Polarization of the T cell also provokes that the centrosome, which is the main microtubules organizing center (MTOC), dissociates from its position near the nuclear envelope and moves towards the contact site T cell-DC. MTOC movement

reorients the microtubule network and the whole cell, bringing the MTOC-associated organelles, such as Golgi complex and the endocytic recycling compartment [75] (Figure 6).

The centrosome (MTOC) is found in the center of the microtubule cytoskeleton and contains the centrioles (barrel-shaped cylinders composed of microtubule triples) and the pericentriolar material (PCM) which is mainly composed by γ-tubulin. The γ-tubulin is a key player in the polymerization of microtubules from α and β- tubulin subunits. Microtubules have a minus end that is proximal to the MTOC and a more dynamic plus end that lengthens away. Microtubule stability given by acetylation of the alfa and beta tubules was shown to be important for TCR-mediated polarization[76]. MTOC reorientation is a hallmark of cell polarity in various cellular processes like asymmetric cell division and directional migration.

A study by Combs[77], shows that MTOC polarization is integrated into the TCR signaling through interaction between the dynein and the adhesion-and-degranulation-adaptor protein (ADAP), which might provide a link between the microtubule cytoskeleton, microtubule motor proteins and the actin cytoskeleton through ADAP/VASP actin binding. This hypothetical linkage between plus-end microtubules and the cortical actin cytoskeleton is supported by data where inhibition of microtubule polarization by colchicine, induced an early retraction of the actin-based protrusions in T cells before IS formation[68] .

Another event that makes part of T cell polarization is the formation of the distal-pole complex. An actin-rich structure which is thought to have a role for pulling away and sequestering negative regulators from the TCR activation complex, and might be required for distinguishing the fate of recently activated T cells into memory or effector[79].



A **B**

Figure 6. T cell polarization upon IS formation. A) Upon TCR recognition, the T cell reorient the MTOC beneath the contact region with the DC. MTOC polarization is commanded by signaling coming from the IS. A complex that consists of adhesion- and degranulation-adaptor protein (ADAP) and the microtubule motor dynein is thought to "grab" the MTOC. Microtubules have been shown to anchor to the pSMAC during TCR engagement. Moreover, microtubule plus-end complexes are proposed to link the microtubule cytoskeleton to the cortical T-cell actin cytoskeleton, thereby providing additional force for MTOC movement through an unknown mechanism. Histone deacetylase-6 (HDAC6), which is important for microtubule stability, is required for TCR-mediated MTOC polarization. Once the MTOC is polarized, cargo travels along microtubules using minus-end directed movement to the MTOC, which directs secretion towards the DC surface[78]. **B)** Reorganization resulting from T-cell activation leads to the formation of the distal pole complex which is formed by the ERM cytolinkers and interacts with DLG1 in order to recruit away from the SMACs molecules such as CD43 or the P-selectin glycoprotein ligand 1 (PSGL1)[67; 78].

1.2.3.1.3. *T cell activation in vivo*

IS formation has been mainly studied using *in vitro* models which do not consider the gradients, factors and external signals that might affect the contacts between T cells and APCs. In 2002, the first *in vivo* studies showed that in intact LN, T cells are highly motile and DCs are able to scan a high number of them over time. Nevertheless, when DCs are loaded with specific antigen the interactions became stable, with an average duration in the order of hours[80; 81].

Von Andrian and colleagues recently proposed a three-phase model for T-cell–DC interaction *in vivo*. According to this model, antigen-specific interactions of T lymphocytes with DCs are transient between 2 and 8 h following the encounters,

stable between 8 and 24 h, and again transient by 24–36 h. The stable interaction in the 8–24 h period probably corresponds to the organization and maintenance of the IS, and is required for complete T-cell activation[82].

It is likely that *in vivo,* T-cell behavior is strongly influenced by chemoattractant forces in the presence of lymph and blood flow. Since T cell activation is characterized by both transient and stable interactions, it has been proposed that chemokine-mediated signals compete with TCR-mediated stop signals and that the combination of the two types of signals determines the duration of T cell–APC interactions. Studies have demonstrated that the accumulation of chemokine receptors at the T cell–APC contact site requires formation of a productive IS and chemokine (CXCL12 and 5) directed secretion by APCs. In this way, T cell responsiveness to other chemoattractant sources is reduced and stability of T cell–APC interactions is increased[83] (Figure 7).

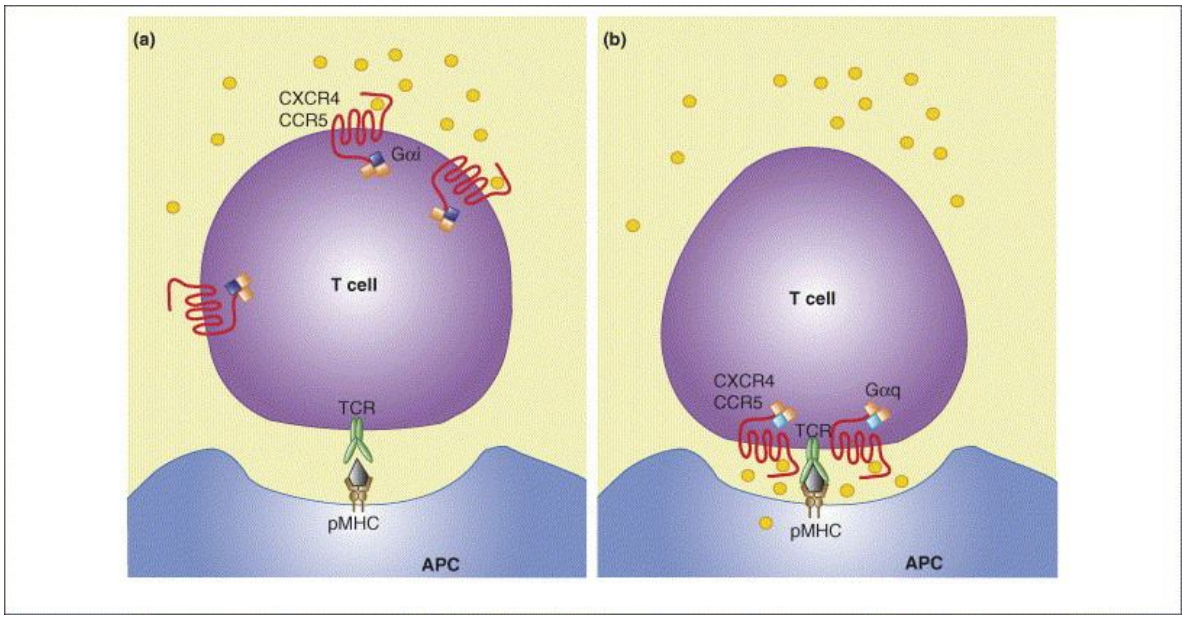


Figure 7. Chemokine receptors in T-DC synapses. Chemokine receptors recruitment to the IS in T cells is mediated by recruitment of chemokines 5 and CXCL12. In DCs represents a mechanism used to reinforce the synapse and avoid early splitting due to external chemoattractant sources, thus, enhancing T cell activation[83].

1.2.3.1.4. *DCs in synapse formation*

Molecular events that contribute to synapse formation and maintenance in T cells have been characterized quite extensively using artificial APC, whereas little is known about the mechanisms controlling the coordinated transfer of different signals from DCs to T cells.

The first evidences of the active role of the APC in the IS were given by Al-alwan et al, who demonstrated that DCs actively polarize F-actin and fascin, during formation of IS with CD4 T cells[84; 85]. Later, it was assessed that DCs rearrange their actin cytoskeleton towards naive CD4+ T cells only in the presence of specific MHC-peptide complexes[69]. Concomitantly, Kondo et al [86] showed that IS could take place in the absence of antigen, inducing TCR signaling and T cell proliferation, probably caused by the density of HLA class II molecules on the surface of DCs in conjunction with the pool of displayed self-peptides. Benvenuti et al. described for the first time the functional role of the maturation state of the DC together with the presence of antigens on IS. Immature DCs were able to establish multiple transient contacts of low stability and with no mature immune synapses taking place. However, they observed that in the absence of antigen, DC maturation induced a minor increase in CD3, LFA-1, and LAT clustering at the immune synapse, but effective clustering, TCR signaling and T cell activation required both DC maturation and antigen recognition [62; 87].

Bloom has documented the role of spinophilin in formation of IS by DCs. This adaptor protein initially found in the dendritic spines of neurons in nervous central system, shows dramatic changes in its distribution accompanying the formation of the immunological synapse. Spinophilin contains a PDZ domain, which is often found in scaffold proteins that bind the cytoskeleton as in T cells, and controls polarity as SCRIB[88](figure 8). The spinophilin null phenotype has a great impact on the triggering of a highly effective immune response.

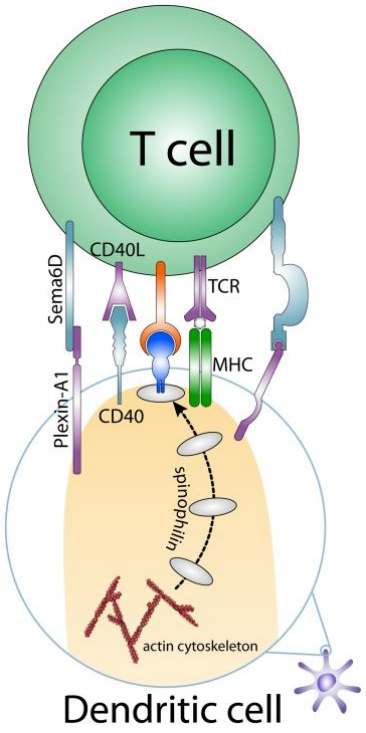


Figure 8. DCs polarization in Immune synapses. In immature DCs, spinophilin is located throughout the cytoplasm but redistributes to the plasma membrane upon stimulus-induced maturation. In DCs interacting with T cells, spinophilin is polarized dynamically towards the IS in an antigen-dependent manner and induce the polarization of adaptors proteins such as the plexins[88].

Another adaptor protein with a shared role in neuronal synapses is plexin-A1. Plexin-A1 belongs to a family of cell surface proteins that are known to act as receptors for semaphorins. In DCs, plexin-A1 appears to be retained in an intracellular compartment, making its way to the cell surface after TNF-α stimulation, where it clusters in a multifocal pattern localizing to the T cell synapse[89].

A recent report revealed an unexpected function of synapse formation in DCs. Riol-blanco et al. demonstrated that CD40 signaling upon IS formation induce ATK1 activation, which inhibits the apoptosis of DCs in stable conjugates with T cells. In parallel, they claimed that soluble factors secreted by both T cell and DC are not enough to increase DC survival[90].

1.2.3.1.5. *Information exchange at the IS*

Factors secreted by lymphocytes are often released into an environment that is densely populated with many cell types, which brings the problem of specificity of intercellular communication. In particular, CD8+ and CD4+ T cells must operate their secretory responses in a targeted way as to avoid activating or killing the wrong cell. First studies of the MTOC, secretory organelles and Golgi in T cells suggested the existence of a mechanism for targeted secretion towards the APC.

Currently, it is established that many hematopoietic cells are able to perform directed secretion. Mast cells and granulocytes polarize their degranulation in response to FcR cross-linking[91], while natural killer cells and cytotoxic lymphocytes direct the content of their secretory lysosomes towards a specific target[92].

Imaging studies have shown that lytic granules are released by cytotoxic T lymphocytes (CTL) at a defined point within the synapse[75]. In particular, it has been shown that CTL first reorient the MTOC upon TCR signaling towards the IS, next the MTOC docks at the cellular membrane and finally the lytic granules are secreted. Recently, it has been shown that MTOC and lytic granule polarization are independently regulated in response to the strength of TCR signaling[93].

In vitro and *in vivo* experiments have shown that several important cytokines, such as IL-2, accumulate beneath the IS in T helper cells after stimulation by an APC[94]. Huse et al have shown that T helper cells use 2 directionally distinct pathways for secretion of cytokines and chemokines. The first one, release directly towards the IS in an antigen specific way molecules such as IL-2, interferon-γ and IL-10. The second is multidirectional and includes the secretion of TNF, IL-4 and CCL3, which seems to be involved in inflammation responses and the mobilization of bystander cells[95].

Ii is very likely that the polarized and the multidirectional secretion pathways are regulated by specific vesicle markers that control their fate after being produced in the Golgi and anchored to the microtubules. This phenomenon was observed by Stow in macrophages, tracing the trafficking markers of different cytokine-containing vesicles[96; 97].

Signals are continuously delivered to the T cell during prolonged interactions with DCs. MTOC reorientation controls the directed secretion of cytokines and chemokines, which might travel along the microtubules using their slow-growing end and then being released towards the engaged DC. Alternatively, stores of IL-2, IL 4 and IL-5 are directed towards the DC after MTOC reorientation[98](Figure 6).

There have been some reports suggesting that also DCs might make use of the polarized secretion. Semino et al showed that in conjugates with NK cells, immature DC increase their free Ca^{2+} concentration, rearrange their cytoskeleton and co-ordinately secret IL-18 towards the interacting NK cell[99]. Borg et al. showed that the formation of specific DC/NK conjugates induces the polarization of the IL-12 toward the synapse and provoke NK cell activation[100]. There is only one report that studies the directed secretion in T cell-DC conjugates, suggesting that IL-1B and cathepsin D are released toward the IS. However, the mechanism and the molecular complexes that support polarization in DCs are not well understood[101].

MTOC polarization has been functionally linked to polarized secretion of cytokines and lytic in immune cells. A list of the proteins linked to these processes and the cell type where they have been studies is summarized in the Table 2.

Table 2 Proteins identified in polarization and secretion pathways in several cell types.

Process	Protein	Cell type
Mtoc Polarization	ADAP	Jurkat T cells[77]
	Dynein	Jurkat T cells[77]
	FMNL1	NIH 3t3[102]
	Rac, Cdc42	NIH 3t3[102; 103]
	Pyk2	Natural Killer (NK)[104]
	Erk	Cytotoxic T lymphocytes (CTL)[105], NK[106]
	HDAC6	CD4 T helper
	WASp	CD4 T[95]
Lytic-granule release	Rab27a	CTL[107]
	AP-3	CTL[108]
	Munc13-4	CTL, Mast cells[109]
	Syntaxin 11	CTL[110]
	Slp1, Slp2	CTL[111]
	Paxilin	CTL[105]
	WIP	NK[112]
Polarized cytokine secretion	Rab3d, Rab19	CD4 T helper[95]
	Syntaxin 4-23	Jurkat T cells[113]
	WASp	CD4 T cells, NK[95]

1.2.4. **Interleukin 12, an example of the three signals integration**

IL-12 is one of the most important cytokines produces by DCs upon TLR engagement. IL-12 is produced by several DC subsets after challenging with different bacterial strains, that stimulates TLRs 3, 4 or 9[114]. IL-12 is a covalenty linked heterodimer formed by a 35 kDa light chain, known as p35, and a 40 kDa heavy chain known as p40. The p35 protein is homologous to other single-chain four-alpha helical cytokines like IL-6 and granulocyte colony-stimulating factor (G-CSF), whereas p40 is homologous to the extracellular domain of members of the hematopoietic cytokine-receptor family (Figure 9)[115].

IL-12 positively regulates its own production via the induction of IFN-gamma, which primes monocytes and neutrophils for further IL-12 production. Conversely, IL-12 production is inhibited by other cytokines including IL-10, IL-11, IL-13 and type I interferons[117; 118].

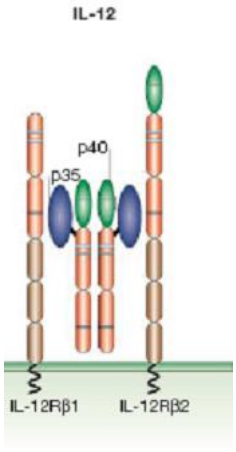


Figure 9. IL-12 structure. IL-12 is an heterodimer composed by a light chain (p35) and a heavy chain (p40). The bioactive form known as p70 binds the IL-12 receptor expressed by T cells[116].

Studies with deficient mice for both IL-12 subunits or for the IL-12 receptor have revealed that IL-12 has an important role in favoring T helper 1 (Th1) response[119]. Both *in vivo* and *in vitro*, IL-12 is required for the optimal differentiation of CD4+ T cells into high-level IFN-γ-producing Th1 cells[120].

Though initially it was believed that IL-12 was sufficient to induce Th-1 cell differentiation, it has been shown that it may be more important for amplifying and fixing the phenotype of already committed Th1 cells than for directly priming naive CD4+ T cells for Th1-cell differentiation[121].

IL-12 is synergistic with CD28 stimulation, and facilitates the T-cell proliferation and IFN-gamma production. In particular, IL-12 enhances the generation and cytotoxicity of T lymphocytes, inducing the transcription of genes that encode cytotoxic granule-associated molecules and upregulating the expression of adhesion molecules[122; 123].

The Il-12 receptor is composed of two chains, β1 and β2 [123]and is mainly expressed by activated T cells, NK, DCs and in low levels by resting T cells. The affinity of IL-12 for either subunit alone is low, but coexpression of both β1 and β2

subunits generates IL-12 high-affinity binding sites. IL-12p40 interacts predominantly with the β1 subunit, whereas p35 interacts largely with the β2 subunit.

The specific effects of IL-12 are caused by ligand-induced autophosphorylation and transphosphorylation of receptor-associated Janus kinases (JAK). JAK activation induces tyrosine phosphorylation of the receptor subunits located in the intracellular domain. These phosphorylated tyrosines serve as docking sites for STATs (signal transducers and activators of transcription) and potentially other signaling molecules. IL- 12 specifically induces the tyrosine phosphorylation and DNA binding of two STAT family members, STAT3 and STAT4 (Figure 10). Their activation has been shown to be necessary but not sufficient to explain the ability of IL-12 to induce Th1 differentiation[124].

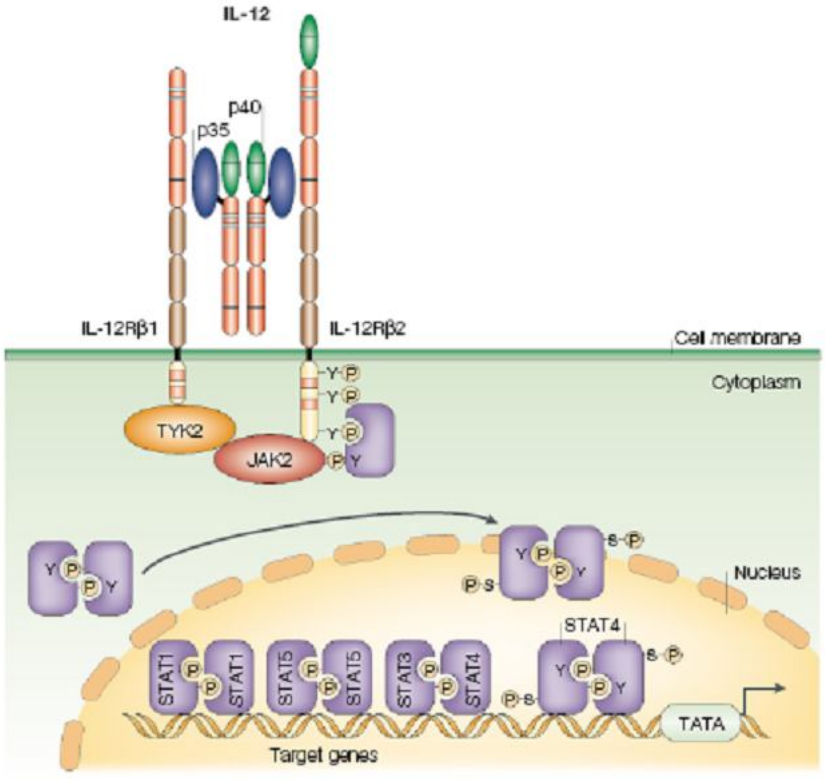


Figure 10. IL-12 receptor signaling pathway The interleukin-12 (IL-12) receptor is composed of two chains, IL-12Rβ1 and IL-12Rβ2. Signal transduction through IL-12R induces tyrosine phosphorylation, primarily of the Janus family kinases JAK2 and TYK2, which, in turn, phosphorylate and activate signal transducer and activator of transcription 1 (STAT1), STAT3, STAT4 and STAT5. The specific cellular effects of IL-12 are due mainly to its ability to induce activation of STAT4[116].

2. MATERIALS AND METHODS

Mice

Six to eight weeks old C57BL/6 females were purchased from Harlan (Milano, Italy). WASp- mice on a C57BL/6 (CD45.2) genetic background were a gift from S. Snapper (Massachussets General Hospital, Boston). GFP-centrin mice were generated from a construct given by Michel Bornens (Institut Curie, Paris) and were a gift from Chantal Desdouets (Institut Cochin, Paris). OVA-specific, MHC class-I OT-I and MHC class-II OT-II, TCR transgenic mice were purchased from the Jackson Laboratories. CD45.1 congenic C57BL/6 (kind gift from Pierre Guermonprez, Institut Curie, Paris) were bred to OT-I mice to obtain OT-I/CD45.1.

Mice were bred and maintained in sterile isolators. 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).

Cells

Bone marrow-derived DCs were differentiated *in vitro* from the bone marrow of the different mouse genotypes using culture medium containing Fms-like tyrosine kinase 3 ligand (Flt3L). DCs were used for experiments between day 7 and 8 when expression of Cd11c was higher than 80%.

For experiments with endogenous DCs, spleens from mice were extracted, homogenized and digested with collagenase D (1,6 mg/ml, Roche) and DNase I (0,1 mg/ml, Roche). Enrichment of DCs was performed by density gradient in an Optiprep solution (Sigma) 1,068 g/cm^3 .The very low density fraction mainly composed by DCs was recovered and subjected to purification using CD11c microbeads (Miltenyi Biotec). OT-I and OT-II cells were isolated from total lymph node suspension by negative selection using MACS® isolation kits.

Antibodies and FACS reagents

The following antibodies for FACS analysis were purchased from BD Pharmingen: FITC and PE-conjugated anti-CD11c, FITC and PE-conjugated anti I-A^b, PE-conjugated anti-CD86, PE-conjugated anti-CD11b, PE-Cy5-conjugated anti-CD8, PE-conjugated anti CD45.1, biotinylated anti-CD69, biotinylated anti-CD3. CFSE (5-(6)-carboxyfluorescein diacetate succinimidyl diester), SNARF and CMTMR were purchased from Molecular Probes.

Bacterial Infection

Salmonella typhymurium strain ATK-GFP was kindly gifted by M. Rescigno (IEO, Milan). Bacteria were grown in LB medium (kanamicin 25 mg/ml + ampicilin 50 mg/ml) until it reached an O.D of 0.6. HEK293 or BM-DCs were infected at different infection ratios in IMDM medium during 1 hour, gentamicin (50μg/ml) was added for an additional hour. Medium was washed away thoroughly and cells were lysed with Triton X-100 (0.5%). The bacteria internalized were released and plated on agar petri dishes with kanamicin + ampicilin. The day after, the number of grown colonies was counted and correlated with the initial number of bacteria. For FACS reading, cells were not lysed after treatment with gentamicin but collected and the intensity of the GFP signal was correlated to the number of bacteria phagocytosed for each DC.

Time-lapse video microscopy (Trajectories)

For the dynamic analysis of DCs trajectories, $3x10^5$ immature or LPS-pulsed DCs (overnight, 10 μg/ml) were plated on fibronectin-coated coverslips and placed into a chamber on a Zeiss LSM510 META Axiovert 200M reverse microscope at 37°C in a 5% CO_2 atmosphere. Transmitted light images were taken with a 63X objective and a 3CCD camera every 30 seconds during 40 minutes. Recording of the trajectories, displacement analysis, and velocity measurements were made using the Image J software. For analysis of the conjugate formation, mature DCs were incubated for one hour with the MHC class-I restricted peptide of ovalbumin 257-

264(SIINFEKL) (0,1 nM) before plating. 1 x10^5 OT-I cells were added to the dish and images were taken starting 5 min after landing on the same plane of DCs. Each DC was analyzed along the length of the movie and the number and duration of contacts established with T cells was scored.

In vivo migration assay

WASp- and wild-type (WT) BM-DCs were harvested at day 7 and labeled with 5-(6)-carboxyfluorescein diacetate succinimidyl diester(CFSE, molecular probes) 2μM, according to manufacturer instructions. After labeling, 5x10^5 or 2x10^6 cells, depending on the experiments, were injected into the footpad of C57BL/6 mice. To quantify the number of migrating DCs single cell suspensions from the draining popliteal lymph node were obtained by digestion in collagenase D at day 1, 2 and 3 post-injection. The absolute numbers of $CFSE^+/CD11c^+$ cells were quantified by FACS by acquiring all cells in each sample.

Immunostaining on lymph node sections

For localization of DCs within lymph nodes C57BL/6 WT mice were injected with 5x10^5 WT or 1x10^6 WASp- CFSE-labeled DCs. Lymph nodes were harvested at day 2 and fixed in paraformaldehyde. Tissues were snap frozen in Tissue-Tek. Frozen sections were fixed in cold acetone and incubated with biotinylated anti-mouse CD3 followed by Alexa-647-conjugated streptavidin. Images were acquired using a LSM 510 Meta using 40 /0.40 NA oil objectives and MetaView 4.6 software (Molecular Devices, Downingtown, PA).

Adoptive transfer and T cell activation

1x10^6 OT-I/CD45.1 cells were purified as described above and injected intra venously into recipient host. For priming with BM-DCs, cells were pulsed with graded dose of the MHC class-I restricted peptide of ovalbumin (SIINFEKL) for 3 hr in complete medium washed and labeled with CFSE. 2x10^5 WT or 6x10^5 $WASp^-$ DCs were injected sub-cutaneously 24 hours after transfer of OT-I cells. At day 3

after DC injection, popliteal draining LNs were collected, digested in collagenase and the percentage of OT-I/CD45.1 cells was evaluated by gating on OT-I/CD45.1. For comparison of the priming ability of DCs in LN, we quantified the number of $CFSE^+$ DCs in each sample (by gating on $CFSE^+$ cells). To analyze the CFSE dilution profile of transferred OT-I cells, T cells were labeled with CFSE and the dilution profile was analyzed by gating on $CD8^+/CD45.1^+$ cells.

Time-lapse video microscopy (MTOC reorientation)

For the analysis of MTOC dynamics of reorientation, $2x10^5$ centrin-GFP DCs pulsed for 5 hours with CpG (1 μg/ml), LPS (1 ng/ml) and SIINFEKL peptide (10 nM). DCs were plated on fibronectin-coated coverslips, placed into a chamber with IMDM medium on a Zeiss LSM510 META Axiovert 200M reverse microscope at 37°C in a 5% CO_2 atmosphere. OT-I cells labeled with the vital dye SNARF (Molecular Probes) were added few minutes before starting the record. Transmitted light and fluorescence images were taken with a 63X objective and a 3CCD camera every 30 seconds for at least 40 minutes. The dynamics of centrin-GFP spots corresponding to the MTOC were tracked frame by frame in every single cell, choosing the plane with the brightest GFP spot. Number of cells that reoriented the MTOC, elapsed time between the establishment of the contact and reorientiation, and duration of the polarized condition were analyzed using the Image J software.

DC-T conjugates formation

The formation of DC-T conjugates was assessed by FACS analysis. $5 x10^5$ DCs activated by TLR agonist for time periods from 0 to 12 hours. DCs were pulsed with SINFEKL peptide, stained with SNARF and mixed with CFSE-labeled T cells (1:1 ratio). Green/red doublets were quantified by FACS after 20 min of interaction at 37°C. Data were expressed as percentage of T cells engaged in doublets over the total number of T cells.

Immunocytochemistry

DCs were stimulated at different time points with CpG (1 μg/ml), LPS (1 ng/ml), pulsed with graded doses of SIINFEKL peptide and transferred to slides coated with fibronectin (Sigma-Aldrych, 10 μg/ml). For synapse formation, OT-I or OT-II cells were added to DCs in a 1:1 ratio and incubated at 37 for 30 minutes. In some experiments, OT-I cells were labeled with CFSE (2 μM). After fixation with 4% paraformaldehyde/PBS, primary and secondary antibody staining was done in PBS/BSA 0,1% /saponin 0,05%. The dilutions for the primary antibodies were: rat α-tubulin (1:400), IL-12 p40/p70 (1:100), Vb5.1/5.2 (1:100), TNF (1:100), VAMP-7(1:500), cd11c (1:100). Anti VAMP-7 antibodies were a kind gift of Thierry Gally (Institut Jaques Monod, Paris), anti-tubulin antibody was purchased from AbD Serotec, all the other antibodies were purchased from BD-Pharmingen. The secondary antibodies were mouse Alexa-647, rat Alexa-488, rat Alexa-555, rabbit Alexa-555 from Molecular Probes. Phalloidin-Texas red (Sigma) was used to detect polymerized F-actin. Confocal images were acquired in a LSM510 META Axiovert 200M reverse microscope with a 63x objective. Z-projection of slices, 3D and image analysis were performed using Zeiss LSM image examiner and image J. At least 30 conjugates for slide were analyzed in at least three independent experiments.

Analysis of polarization

The analysis of polarization was performed on individual DCs in contact with a single T cell. This was the most represented condition in our experiments. To score conjugates with polarized MTOC, we calculated the ratio between the DC diameter and the distance of the MTOC to the synapse region. Conjugates in which such value was lower than 0,3 were considered as “polarized”. We defined cytokine-containing vesicles using a standardized threshold calculated with Image J on Z-projections of confocal sections. The distance between the MTOC and all vesicles was measured on individual cells and plotted as average distance in at least 30 cells/condition. We measured the distance between the synapse region and each cytokine vesicle. The ratio between the mean distances of cytokine

vesicles and synapse region/diameter of the DC was calculated. The cytokine was considered polarized when this ratio was lower than 0,3. For the WASp- DCs, polarization was assessed on cells with IL-12 signal intensity comparable to the observed in WT DCs.

ELISA

7,5 x10^5 DCs (WT or WASp-) were stimulated with CpG/LPS for different periods. At the end of the incubation period the cell culture supernatant was harvested and the cell pellets washed 2 times in PBS and lysed in 120 μl of TNN + 1 μl of protease inhibitor cocktail(PIC). The levels of IL-12p40, IL-12p70 and TNF in the supernatants and lysates were determined by commercial ELISA kits (Bd Biosciences and eBioscience) according to the manufacturer instructions.

Cytoskeletal disruption

To inhibit microtubule polymerization cells were treated with colchicine or colcemide (SIGMA) (1 μg/ml) for the last 5 min of the pulsing period with TLR agonist. The cells were extensively washed before mixing to T cell to avoid carry over of the drug.

STAT4 phosphorylation

For analysis of STAT4 phosphorylation by Western Blot 2x10^5 DCs were mixed to 4x10^6 OT-I in a 96 wells plate by spinning at 800 rpm for 1 min. After 30 min of incubation at 37°C, the cells were lysed and cell lysates resolved by 10% SDS-PAGE. PDVF membranes were blocked with TBS/BSA 5% during 30 min and incubated with of Rabbit-anti-pSTAT4-ser721 antibody (SantaCruz) (dilution 1:500) followed by an anti-rabbit-HRP antibody (Sigma) (dilution 1:5000).

For FACS analysis, DC-T synapses were formed as above. T cells were incubated with supernatant of TLR stimulated DCs as a control (referred to as soluble IL-12).

At the end of incubation at 37°C cells were fixed with 1% PFA (10 min) and permeabilized with methanol 80% (20 min). After washing, cells were stained with pSTAT4-FITC or mouse IgG isotype control (Pharmingen, 1:25), CD45.1-rhodamine (1:400) and CD8-Cy5 (1:400). For FACS analysis, cells were gated on CD8-CD45.1 double positive events and inside this population, T cells alone or conjugated with DCs were distinguished by the FSC/SSC profile.

Statistical Analysis

All data were reported as the mean ± standard error mean (SEM) as calculated using GraphPad Prism 5 software. The unpaired student *t* test was performed as indicated in the text to assess significance.

3. RESULTS

WASp knockout (WASp-) mice proved to be a valid model to study cellular functions and gene therapy approaches[20; 125; 126]. For our studies we used a WASp knockout mouse model on the H-2b C57/BL6 background that has been a generous gift from Scott Snapper. Since the WAS gene is on the X chromosome, we used throughout the study WAS-/- homozygous female or WASp- males and wild type (WT) littermate as control. Briefly, the strategy used by Snapper to disrupt the murine WASP gene consisted in gene-targeted mutational techniques to insert a neomycin-resistance gene (neo) into exon 7(figure 11).

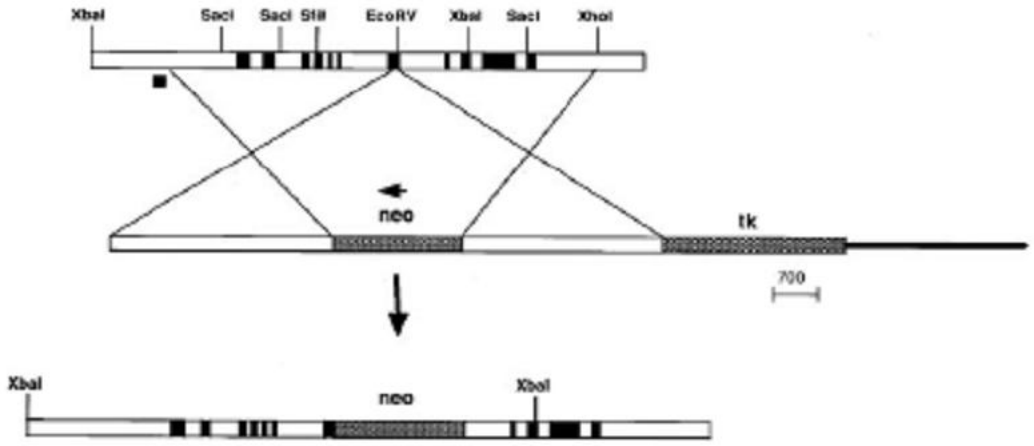


Figure 11. WASp deficient murine model. The WASp gene was disrupted by homologous recombination with the neomycine-resistance gene[5].

Mice genotyping by PCR was performed using specific primers (see Materials). A 889 bp band corresponding to the *neo* inserted gene was observed in the WASp- mice, whereas a 457 bp band, corresponding to the WAS gene was observed in WT mice. Absence of WASp was further confirmed by Western blot analysis and intracellular FACS (not shown) in randomly chosen mice (Figure 12).

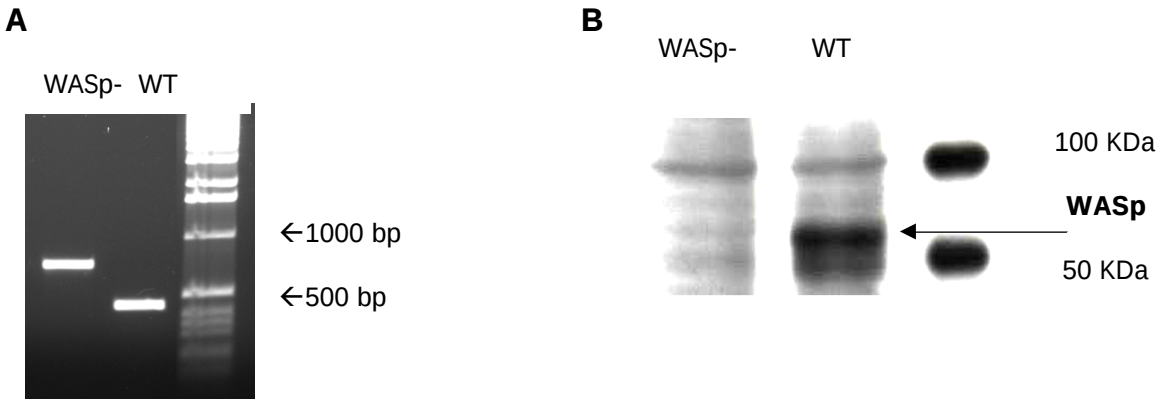


Figure 12. Genotyping of WASp mice. A) PCR products after amplification with specific primers from DNA samples obtained from WT or WASp- genomic DNA were runned by electrophoresis in agarose gels. 889 bp band corresponding to the *neo* inserted gene was observed in the WASp- mice, while a 457 bp band, corresponding to WASp gene was observed in WT mice. **B)** Lysates obtained from spleen cells were resolved by SDS 10% and Western blotting with WASP-specific antibodies. A 62 kDa specific band is observed only in WT cellular extracts.

Maturation of WASp- DCs

We first evaluated the capacity of DCs from WASp- mice to differentiate and mature after TLR stimulation. To this aim BM differentiated WASp- and WT DCs were stimulated for six hours in the presence of CpG and LPS, stained with CD86 (a costimulatory molecule associated with maturation in DCs) and cd11c antibodies and analyzed by FACS. The percentage of $CD11c^+$-$CD86^+$ in immature DCs was 11,50 and 10,18 for WASp and WT respectively. Upon stimulation the percentage of $CD11c^+$-$CD86^+$ cells raised up to 71,48 and 65,84 for WASp- and WT respectively (figure 13). This result indicates that maturation upon TLR stimulation is not affected by the lack of WASp in DCs.

Phagocytic defect in WASp- DCs

We next moved to analyze the ability of WASp- DCs to uptake pathogens by phagocytosis. Different models have been used to check the internalization capacity of dendritic cells. We used as a model a *Salmonella typhimurium* (ATK)

strain that is defective for the TTSS (Type Three Secretion System) and thus rely exclusively on the endocytic mechanisms of the infected cell[127]. To detect bacterial internalization by immunofluorescence and FACS analysis we used a strain containing the GFP protein (ATK-GFP hereafter).

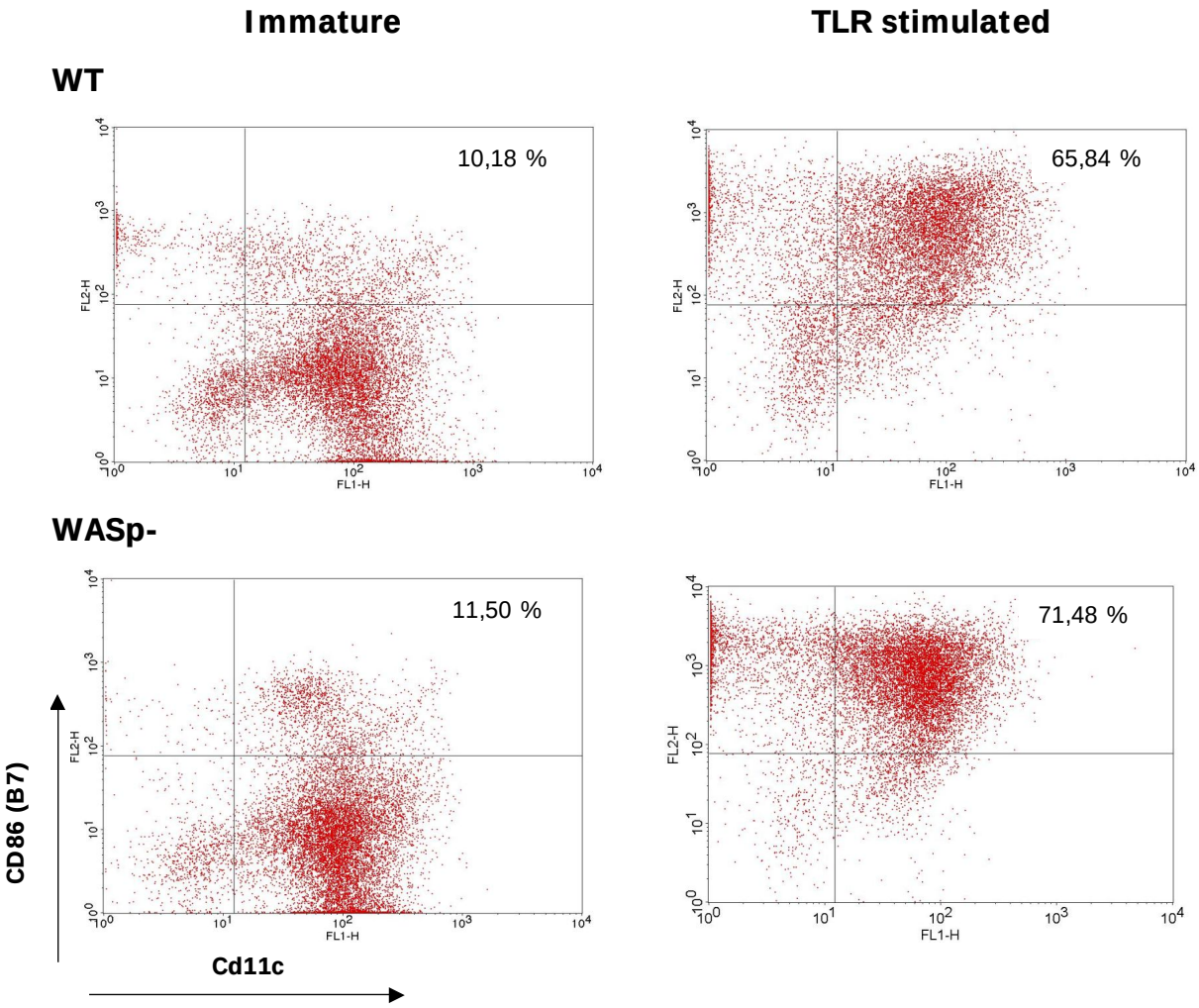


Figure 13. Maturation profile of WASp- DCs. WT and WASp- BM-derived DCs were collected on day 7 and were stimulated or not with CpG and LPS during 6 hours. Cells were marked with CD11c (x axis) and cd86(y axis) antibodies and analyzed by FACS. Dot plots show that both populations have practically the same percentage of DCs $CD11c^{+}$-$CD86^{+}$ before and after TLR stimulation.

Cells were pulsed with *Salmonella ATK* for one hour in culture medium without antibiotics. Gentamicin was then added for an additional hour to kill the remaining bacteria outside, whereas the phagocytosed bacteria were protected. A quantification of the *Salmonella* ATK-GFP ingested by the cells was done by

measurement of the fluorescence by FACS or by colony counting, after plating the internalized bacteria on Petri dishes (see Materials).

We first set up the incubation times, ratio of bacteria for each DC and the dose of gentamicin required to inhibit bacterial growth after one hour. Then, we validated our model and checked if internalization was caused by phagocytosis and not by autonomous bacterial infection. HEK293 cells (with no phagocytic activity) or WT BM-derived DCs were pulsed with *Salmonella* ATK-GFP (ratio 1:10) and green fluorescence was measured by FACS. We observed that after 1 hour of infection 20% of WT DCs have internalized at least one fluorescent bacterium. In contrast, HEK293 cells had no GFP signal inside (Figure 14). This result confirmed that the ATK-GFP strain is defective for autonomous infection under our working conditions.

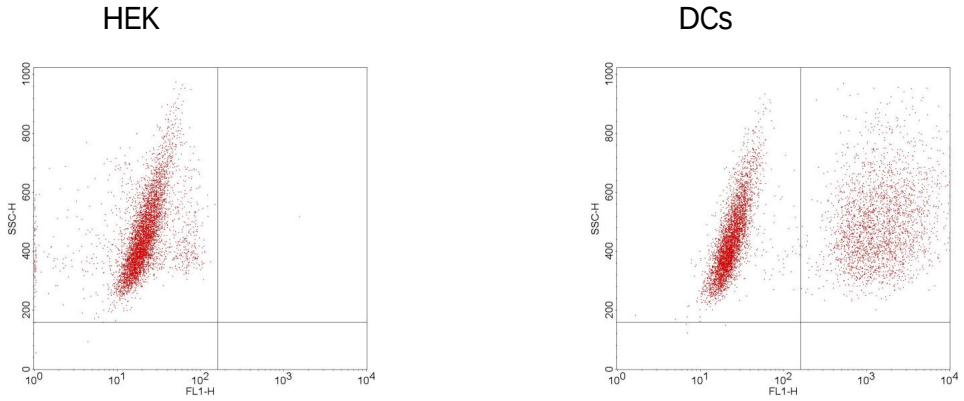


Figure 14. *Salmonella* ATK-GFP is impaired for autonomous infection. $1x10^5$ HEK293 or WT BM-derived DCs were pulsed with $1x10^6$ *Salmonella* ATK-gfp bacteria, after 1 hour medium was extensively washed and green fluorescence was read by FACS. While no HEK293 cell internalized any bacteria (left panel, right quadrant), 20 % of BM-derived DCs phagocytosed at least one bacterium (right panel, right quadrant).

Next, we proceed to evaluate the efficacy of *Salmonella* internalization by WASp-DCs. $1x10^5$ WT or WASp- BM-derived DCs were pulsed with *Salmonella* ATK-GFP bacteria (ratio, 1:10). Gentamicin (50 μg/ml) was added after one hour of infection and left for an additional hour. Medium was extensively washed away and DCs were lysed. Exposed bacteria after DC lysis were plated on Petri dishes and the number of colonies grown was correlated to the number of bacteria phagocytosed by DCs. A series of six independent experiments with at least two

mice for conditions, and triplicates for infection and bacteria plating were performed. The difference on the number of bacteria internalized by the two populations of DCS was plotted as the mean percentage of reduction in the number of bacteria internalized by WASp- DCs with respect to the WT DCs (Figure 15). We observed a decrease on the *Salmonella typhimurium* uptake by WASp- DCs that corresponded to 42.1 +/- 6 %.

To extend this result to a physiologic population of DCs, we performed the same assay using endogenous DCs. The CD11c fraction from spleens of WT and WASp- mice was purified and pulsed with *Salmonella* ATK-GFP (ratio1:10). Gentamicin (50 μg/ml) was added after one hour and left for an additional hour. Medium was extensively washed away and DCs were lysed. The bacteria inside were plated on Petri dishes and the number of colonies grown was correlated to the number of bacteria phagocytosed by DCs. Two independent experiments were perfomed with triplicates of colony plating. We observed again a clear defect on the *S. typhimurium* uptake by endogenous WASp- DCs that corresponded to the 44.6 +/- 6.5 % (Figure 15). These results indicate that lack of WASp significantly reduces the phagocytic capacity of DCs.

We next asked whether bacteria internalized by WASp- DCs, besides being reduced in number, were delivered to the same intracellular compartment in WT and WASp- DCs. To this aim DCs were stained with specific markers of early endocytic vesicles after infection. $1x10^5$ WT and WASp- DCs were pulsed for one hour with $1x10^6$ *Salmonella* ATK-GFP. After infection cells were fixed and stained with LAMP-I, EEA1 and phalloidin (to detect F-actin). Z-projection images were obtained by confocal microscopy. Image analysis revealed that a single DC may uptake more than one bacterium. The bacteria were surrounded by intracellular compartments positive for LAMP-I and EEA-1 markers. However, no apparent difference in the intracellular distribution of bacteria between WASp- and WT DCs was observed (figure 16).

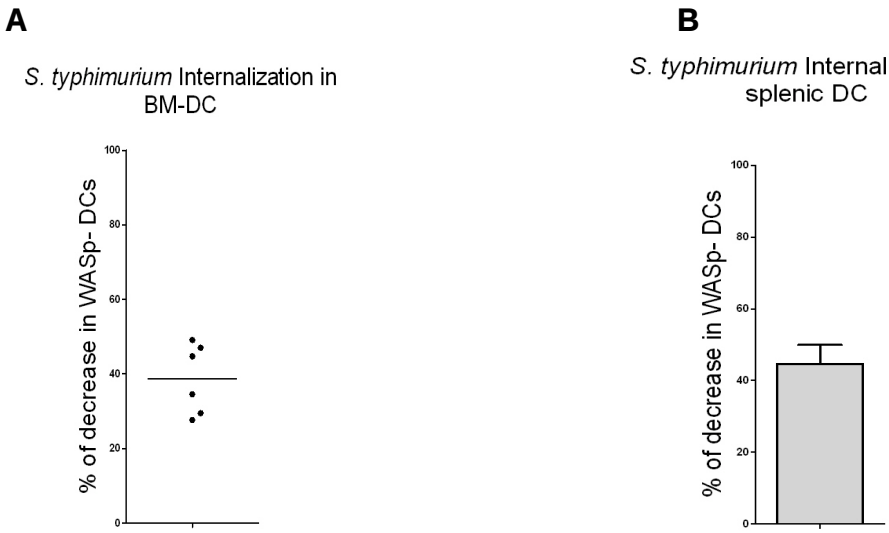


Figure 15. Phagocytic defect in WASp- DCs. A) 1x10^5 WT or WASp- BMDCs were pulsed with *Salmonella* ATK-gfp (ratio 1:10) during one hour, gentamicin was added to the culture medium. After one hour medium was washed away several times and then cells were lysed. Exposed bacteria were plated on Petri dishes and colonies were counted the day after. The percentage of defect in *Salmonella* ATK-GFP uptake by WASp- DCs respect to WT DCs is plotted. Each point corresponds to the mean defect of a series of six independent experiments. **B**) Spleen DCs purified from WT or WASp- mice were pulsed as in (A). Colony counting confirmed the impairment of WASp- seen using BMDCs. Two independent experiments were performed with triplicates for colony plating. Mean of the defect +/- SEM is plotted.

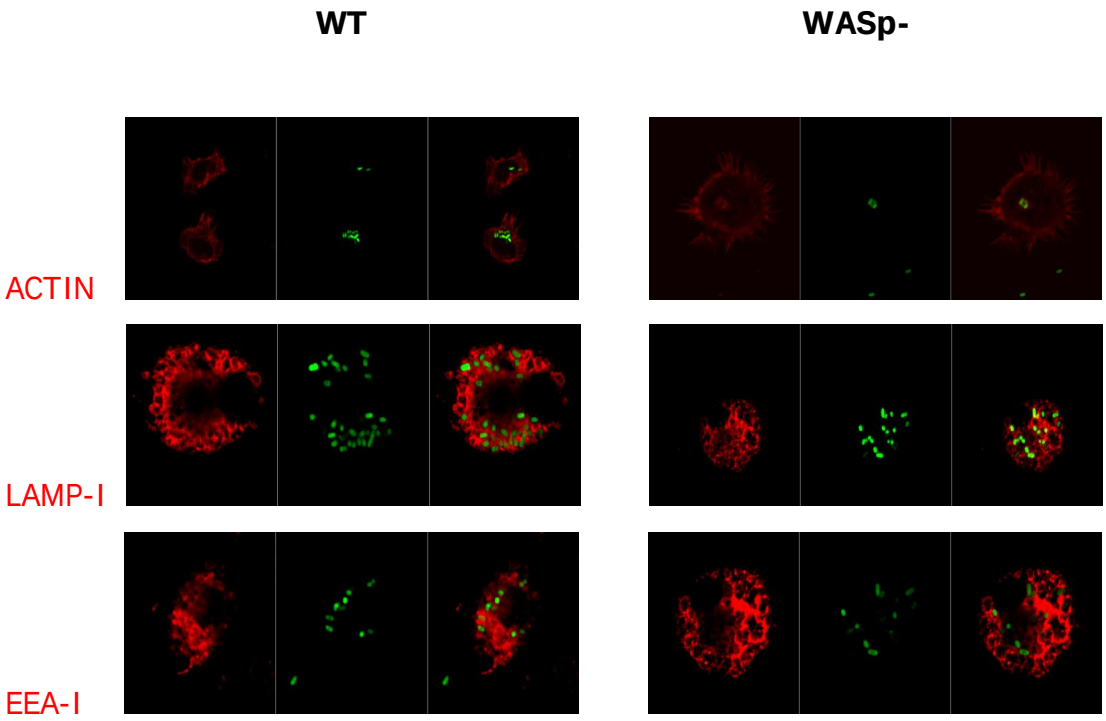


Figure 16. Intracellular Salmonella localization in DCs. WT and WASp- BMDCs were pulsed with *Salmonella* ATK-gfp during one hour, cells were fixed and stained with phalloidin (actin), LAMP-I and EEA-I antibodies (red). Demonstrative Z-projections of single cells revealed no difference in the bacterial (green) intracellular localization.

From this analysis we conclude that WASp- DCs are impaired in the ability to uptake bacterial antigens (45% of reduction). However, our preliminary data indicates that once internalized, bacteria reach the same endocytic compartment.

Role of WASp on DC displacement and migration

After we assessed that WASp deficiency affects bacterial phagocytosis by DCs, we next moved to study its role in other DC functions. It has been shown that during the initial phases of T cell priming, DCs project polarized membrane extension that facilitate the formation of DC-T cell conjugates. This activity is regulated by small GTPases of the Rho family [55]. Since WASp drives actin polymerization downstream of Rho GTPases, we investigated whether WASp deficiency in DCs affects the ability to displace, find naïve T cells and establish stable contacts.

DC trajectories in vivo and in vitro

We first evaluated the ability of DCs to migrate *in vitro* by time lapse video microscopy. BM-derived WT or WASp- DCs were plated on fibronectin-coated chambers. Time-lapse movies of DC movements were recorded during 30 minutes (See materials). We first tracked random movements of WT and WASp- immature DCs. Immature WASp- DCs displayed an altered morphology, failed to extend a polarized leading edge and to retract the rear (Figure 17) As a consequence, the speed of cell migration was reduced as compared to WT cells (WT=0.058 ± 0.003 μm/sec (n=54), WASp- =0.032 ± 0.002 μm/sec (n=46); p<0,001) (Fig 17b) (supplementary movies 1 and 2)..

To compare the morphology and dynamics of mature DCs, cells were stimulated with LPS overnight and plated on fibronectin-coated chambers. Under the microscope we observed reduced morphological differences between WT and WASp cells since ruffling and dendrites extensions were present in both cases (Figure 17a). Migration velocities were increased by LPS treatment, but despite

apparent rescue of morphological abnormalities WASp- cells remained slower than WT cells (WT= 0.080 ± 0.0052μm/sec (n=36), WASp-=0.059 ± 0.0042 μm/sec (n=31); p<0,01) (Figure 17b) (supplementary movies 3 and 4).

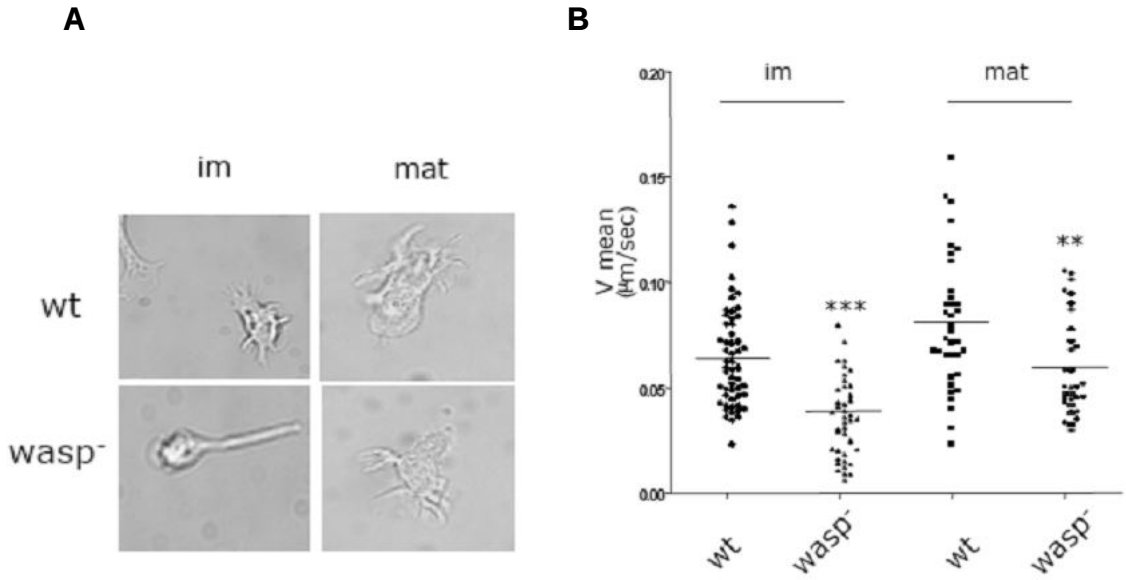


Figure 17. Dendritic cells motility. A). WT or WASp- BMDCs were stimulated or not with LPS, plated on fibronectin-coated chambers and recorded by time-lapse video microscopy. Snapshots show that immature (im) WASp- DCs have a defect to retract the dendrites after migration. A similar ruffling pattern is observed in both mature (mat) populations. **B).** BM-derived DCs were recorded for 40 minutes on fibronectin-coated chambers. The mean velocity of displacement was measured for WT and WASp- DCs. The graph shows mean velocities of cells analyzed in three single experiments, where each point corresponds to a single cell. T-student test was used to assess statistical significance. (P<0,01)

These results indicate that lack of WASp reduces the ability of DCs to travel on a fibronectin matrix, probably because an impairment in dendrites retraction. The impairment is independent on the maturation status of the DCs. This confirms previous published data from Thrasher using a similar model[20].

We next moved to examine if altered DC mobility affects the ability to form conjugates with naïve T lymphocytes. To this aim, we recorded time-lapse movies of the first phases of DC-T cell interaction during priming. To specifically detect the role of WASp expression in DCs, we studied synapse formation using $CD8^+$ T cells of wild type origin. In order to bypass the defective antigen uptake

previously shown (figure 15), cells were pulsed with processed peptide, ready to be loaded on the MHC complexes.

WT and WASp- BMDCs were matured by LPS treatment and pulsed with the SIINFEKL peptide or left un-pulsed. Naïve OT-I cells were added to DCs at 1:1 ratio and differential interference images were collected every 30 seconds for the first 40 minutes of the co-culture. Figure 18a illustrates an example of WT or WASp- DCs in the presence of OT-I cells. The majorities of WT DCs flap their dendrites in all directions and polarize to trap T cells as soon as they arrive in close proximity. However, the cell body of a proportion of WASp- DCs remained anchored to the substrate and failed to trap and establish tight contacts with nearby T cells (Supplementary movies 5 and 6). We quantified the percentage of DCs that established a contact that last more than 20 minutes with at least one naïve T cell. As shown in Figure 18, both WT and WASp- DCs establish only few long contacts in the absence of OVA specific peptide (WT=13,7; WASp-=14,5). Addition of 0,1 nM of OVA peptide induced a high proportion of WT DCs to form long-lasting contacts with antigen specific T cells (47±0,13). In contrast, peptide loading on WASp- DCs induced only a modest increase in the percentage of long-lasting interactions (21±0,15; $p<0,001$). To confirm these observations we moved to examine DC-T cell conjugates using endogenous $CD11c^+$ cells isolated from lymph nodes (Figure 19 d). The overall duration of antigen specific DC-T cell interaction was significantly decreased also in the case of freshly isolated WASp- DCs (WT=18.58 min ± 1.412; WASp-=13.05 ± 1.738, $p<0,05$).

Collectively these results indicate that WASp expression in DCs is required to optimize the encounters and to establish stable interactions with naïve T cells. However, these results were only valid for an *in vitro* model, in the absence of chemotactic signals and in a restricted space where the T cells and the DCs where in close proximity. Thus, we moved to an *in vivo* model, and we asked whether the ability of DCs to migrate from periphery to lymphoid organs, and once there, their capacity to form stable synapses and activate naïve T cells was affected by the absence of WASp.

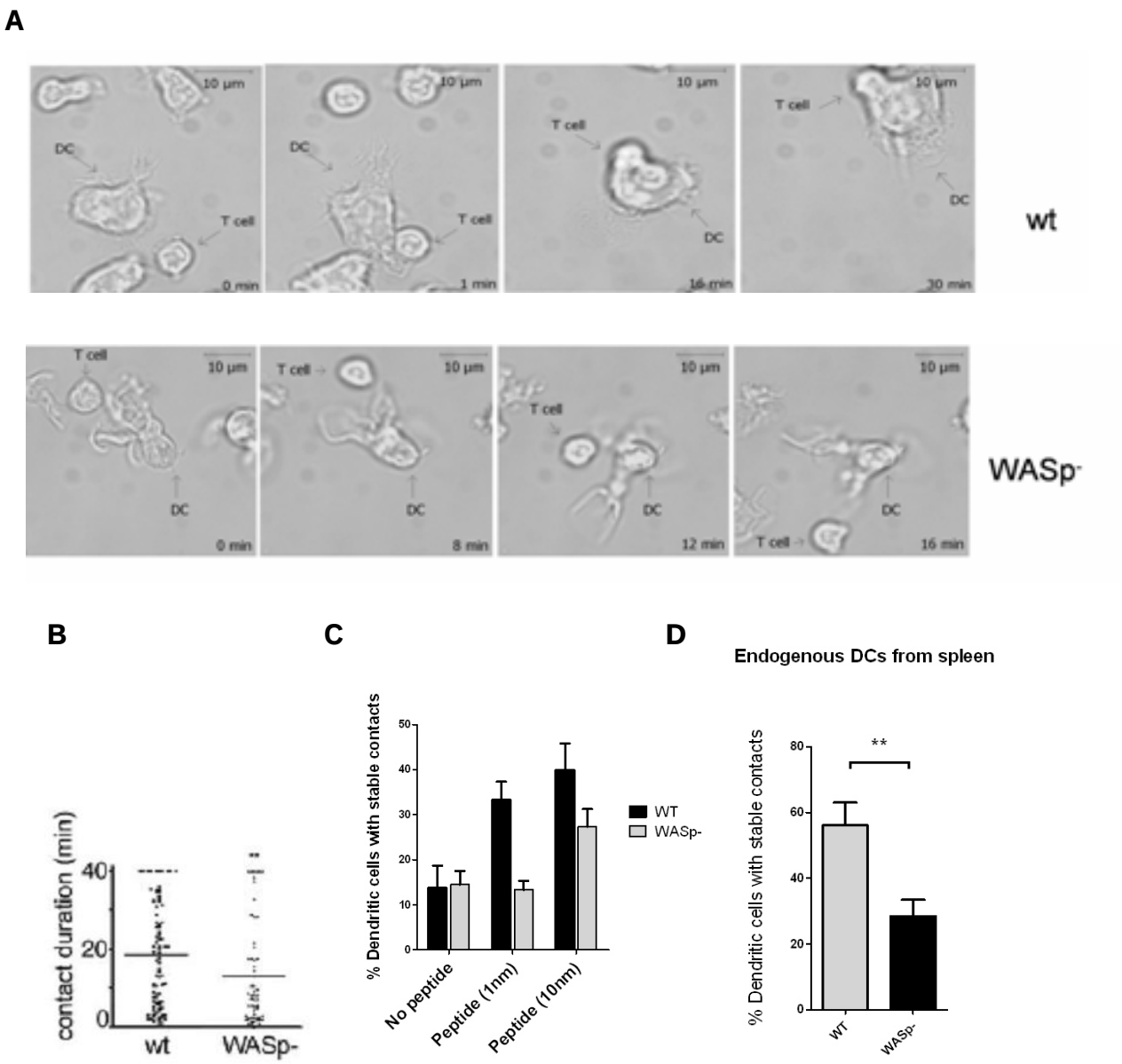


Figure 18. Dc-T cell stable contact formation. A) BM- derived DCs were matured, loaded with OVA peptide and cocultured with OT-I CD8 T cells on a fibronectin matrix. Interactions were recorded during 40 minutes. Snapshots of time-lapse videos show that WT DCs establish stable conjugates with the T cells, while WASp- DCs contact T cells in a transitory way. **B)** Duration of the contacts between a single T cell and a WT or WASp- DC were measured in at least 60 conjugates. Each point corresponds to a single conjugate DC-T cell. The pool of 5 independent experiments is plotted ($p<0,01$) **C)** WT and WASp- mature BM- derived DCs were loaded with increasing doses of peptide and co-cultured with OVA specific T cells. The percentage of single contacts lasting more than 20 minutes was scored. Mean percentage +/- SEM of three independent experiments is plotted. **D)** Endogenous WT and WASp- DCs were purified, stimulated with LPS and loaded with OVA peptide. DCs were mixed with OVA specific T cells and recorded for 40 minutes. DCs that established stable contacts for more than 20 minutes were scored. Bars indicate mean percentage +/- SEM of DCs with stable contacts and are representative of a series of three independent experiments ($p>0,01$).

To this aim, we first examined trafficking of adoptively transferred BM-derived DCs to sites of immune induction. BM-derived WT or WASp$^-$ DCs labeled with CFSE were injected into the footpad of WT recipient animals to analyze the kinetics of arrival to draining lymph nodes. After one day, the number of WASp- DCs that reached the draining LN was decreased by more than 3-fold as compared to WT cells. At day 2 and 3 we found a similar reduction in the number of recovered WASp- DCs indicating that migration is not simply delayed (Figure 19 a). When we increased the DCs input dose by two and fourfold, we observed a linear increase in the number of recovered DCs. However, the inhibition of at least twofold in migration of WASp- cells was maintained along the entire range of doses tested (Figure 20b). Thus, lack of WASp expression in DCs causes a two-fold reduction in migration from the injection site to the draining lymph node.

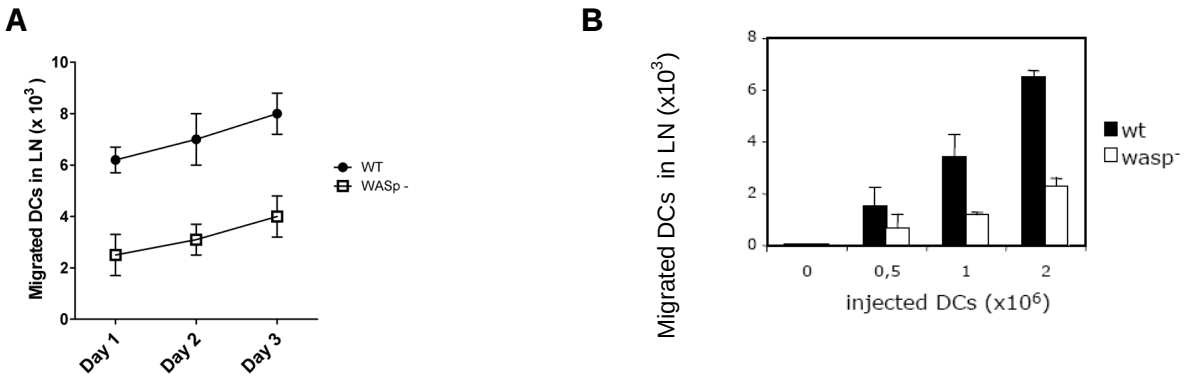


Figure 19. Impaired migration in vivo. A) 5x10^5 WT or WASp$^-$ DCs BMDC labeled with CFSE were injected into the footpad of WT recipient animals to analyze the kinetics of arrival to draining lymph nodes. A reduced amount of WASp- DCs recovered in the LN was observed during the following three days. **B)** Increasing amount of WASp- and WT DCs were injected on the footpad of WT recipient mice and recovered on the LN after one day. Mean of migrated DCs +/- SEM is plotted.

Since we wanted to study the intrinsic ability of WASp- DCs to prime naïve T cell we looked for a way to bypass homing defects. To this aim, we titrated the number of input DCs necessary to achieve comparable numbers of WT and WASp- DCs in lymph nodes. This was achieved at low DCs doses by injecting 2x10^5 WT DC and 6x10^5 WASp- DCs (Figure 20a). Once we managed to adjust the migration defect we examined in detail the regions where the DCs localized inside the LN. WASp- (CFSE labeled) and WT (SNARF labeled) DCs were injected

simultaneously into the footpad of a WT recipient. After one day, LNs were extracted and snap frozen. LN sections were stained with CD3, B220 and PNAd antibodies. These are specific markers that respectively define the T cell zone, B cell folicles or endothelial venules inside the LN. After fluorescence analysis we found that WASp- DCs that have migrated to LN homed to T cell areas similarly to WT DCs (Figure 20b).

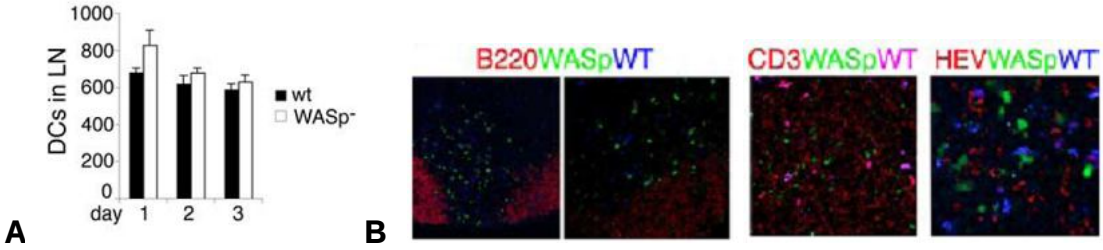


Figure 20. Rescue of defective migration. A) The defect on migration from the footpad to the LN was recovered injecting $2x10^5$ WT DC and $6x10^5$ WASp- DCs, after the three following days, equivalent numbers of CFSE stained DCs were recovered. **B)** LNs of recipient mice were immunostained with CD3, B220 and PNAd (HEV) antibodies to evaluate the localization of WT and WASp- DCs injected. Both populations migrated to corresponding areas in the inner layers of the LN.

Naïve T cell priming

Once established that WASp- DCs normally home to T-cell areas we moved to evaluate the priming of naïve T cells. $2x10^5$ WT and $6x10^5$ WASp- BMDCs were left un-pulsed or pulsed with two different doses of OVA peptide and used to immunize WT hosts that were adoptively transferred with OT-I cells (CD45.1 congenic). LN near the injection sites were harvested after 3 days. Proliferation and accumulation was evaluated by FACS. As shown in figure 21a, the ratio between OT-I/$CD45^+$ (adoptively transferred) over the endogenous $CD8^+$ T was higher in LN of mice injected with WT DCs. This result indicate that despite equivalent number of WASp- DCs in the LN, OT-I cell expansion was significantly inhibited in mice immunized with WASp- DCs.

To get insight into the mechanism of defective T cell accumulation induced by WASp- DCs we analyzed the CFSE dilution profiles of transferred OT-I (Figure 21).

Immunization with $2x10^5$ WT DCs loaded with 0,01 nM OVA peptide was already sufficient to induce up to 40% of T cells to enter division and 30% of the cells to undergo more than seven cycles of division (fully divided cells). After immunization with $6x10^5$ WASp- DCs at the same peptide dose most OT-I cells primed remained undivided (60%) and a little proportion underwent two to seven division (13%). At 0,05 nM WT DCs induced all OT-I to enter the cell cycle and a large proportion of fully divided cells to accumulate (56%). Interestingly, priming with WASp- DCs loaded with 0,05 nM of peptide caused up to 70% of OT-I cells to enter division but the cells remained trapped between 2 to 7 cycles with very few cells beyond the 7^{th} division (13%).

To rule out the possibility that inefficient priming was due to impaired DCs maturation in WASp- DCs[128], we measured by FACS the maturation profile of DCs before and after homing to LN. WT and WASp- DCs were injected into the footpad of WT recipient mice and cells were recovered at day 3 after injection. DCs were stained with maturation markers, MHC-II and CD86, before and after infection and populations were analyzed by FACS. We found no differences in the maturation profile between WT and WASp- DCs recovered in the LN (Figure 21d).

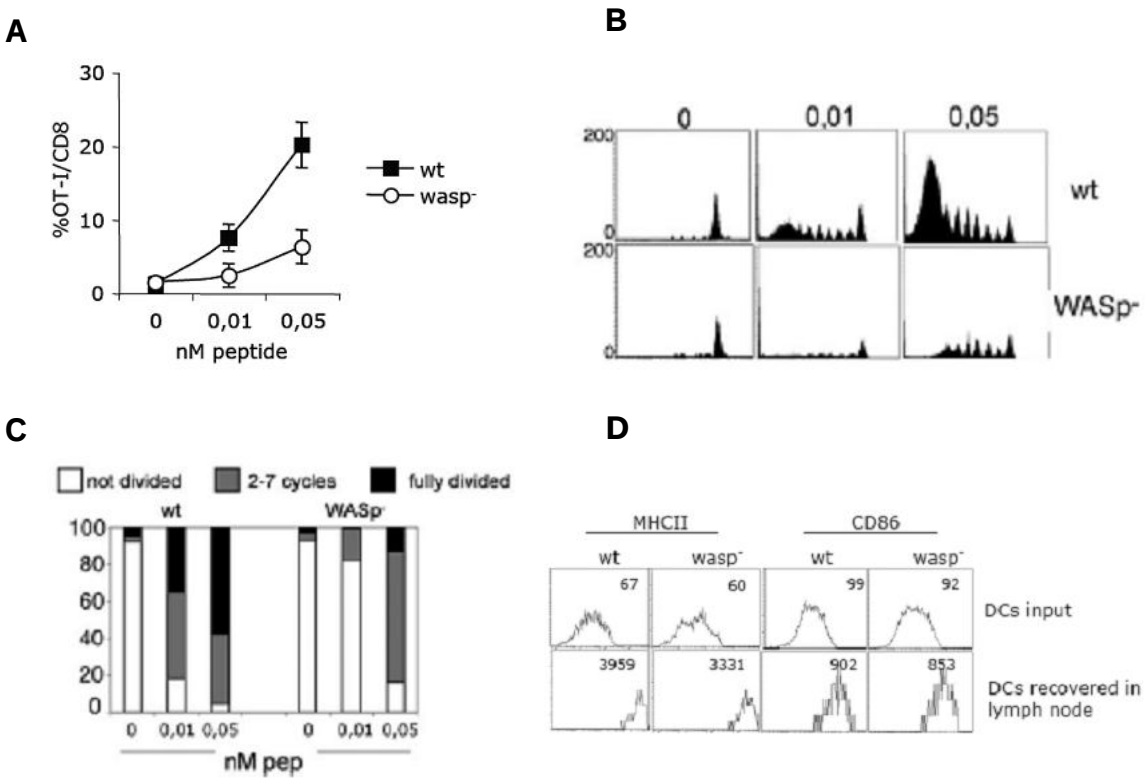


Figure 21. Defective priming of naïve T cells A) Mice were adoptively transferred with CD45.1, CFSE labeled OT-I cells. T cell priming was induced by injection of $2x10^5$ WT or $6x10^5$ WASp- DCs loaded with the indicated doses of MHC class-I OVA peptide. Percentage of OT-I cells over the total $CD8^+$ population at day 3 post-immunization (gated on CD45.1/$CD8^+$ cells). Values are the means ± SEM of four mice per group. **B)** The histogram plots show the CFSE dilution profile of transferred OT-I (gated on $CD8^+$/$CD45.1^+$) in draining LN at day 3. Values are means± SEM of 4 mice per group. **C)** Percentage of OT-I cells that remained undivided (undivided), that underwent 2 to 7 division (2 to 7) or that fully diluted CSFE (fully divided) was plotted. **D)** Mean fluorescence histograms show the staining profiles of MHC-II and CD86 (maturation markers) in WT and WASp– DCs before injection (input) or recovered at day three after injection (DCs recovered in LN).

The above results indicate that DCs require WASp expression in order to induce efficient T cell proliferation when transferred into a host. Thus, WASp expression in DCs is important to initiate $CD8^+$ T cells responses at two levels: by promoting the migration to draining LN and by supporting efficient T cell activation.

In vitro DC-T cell interaction

Our data on T cell activation indicate that WASp- DCs do not efficiently prime naïve T cells in LN. As we have shown, this in part due to impaired ability to form DC-T conjugates. However, our data suggest that other factors may contribute to the strong reduction in T cell priming. It has been proved that an intact cytoskeleton is required for the formation of stable synapses in a variety of cellular models[129; 130] . Taking into account that WASp is an actin polymerizator, we moved to study the distribution of actin and tubulin cytoskeleton in formed DC-T synapses to understand whether WASp deficiency affects appropriate cross talk at the immune synapse.

To this aim, we examined the structure of the cytoskeleton in DCs during interaction with T cells. BM-derived WT or WASp- DCs were activated by TLRs agonist to induce maturation and were loaded or not with OVA class-I peptide. DCs were then mixed with $CD8^+$ OVA specific T cells (OT-I cells) for 30 minutes, fixed and analyzed by confocal microscopy. To visualize the microtubule cytoskeleton we used immunolabeling with anti-tubulin-α antibodies and to see

the actin we used phalloidin. Additionally, to corroborate that the conjugates observed corresponded to mature IS we checked by immunofluorescence the recruitment of CD3 at the T cell side. CD3 binds to the T Cell Receptor(TCR) and it is a hallmark in the formation of the c- SMAC[64].

Given our previous results, we spun together DC and T cells to overcome defective conjugate formation by WASp- DCs. The number of DC-T conjugates was identical in WASP- and WT cells. WASp- DCs showed that shape and distribution of microtubules looked undistinguishable from those in WT cells.

First analysis by confocal microscopy showed that in a high percentage of WT DC-T conjugates the DC's MTOC was in close proximity to the synaptic membrane respect to WASp- DCs (Figure 22) . Since MTOC polarization, a process widely studied in T cells, has never been described in DCs we decided to evaluate in depth this phenomena. We first evaluate the role of specific peptide recognition by T cells on DCs MTOC polarization. To this aim, WT and WASp- DCs were loaded with increasing doses of OVA peptide, co-cultured with OT-I cells during 30 minutes, fixed and immunostained. The percentage of cells showing polarized MTOC was quantified according to the criteria described before (see Materials). As shown in Figure 22 the percentage of DCs showing a reoriented MTOC depended on antigen dose. Only few DCs (5,3%±0.3) were polarized in the absence of peptide, a figure that increased to 20%±2.02 and 42% ±1.8 at 1 and 10 nM peptide respectively. MTOC polarization in WASp- DCs did not response to the increase of peptide dose. Thus, we found a remarkable reduction in the percentage of WASp- DCs with the MTOC facing the DC-T contact region (Up to 70 % of reduction with respect to WT cells at the highest peptide doses, $p<0.01$) (Figure 22b)

Thus, DCs engaged in antigen specific synapses reorganize the microtubules cytoskeleton by redirecting the MTOC towards the interacting T cell in an antigen dose dependent manner, a process where the presence of WASp is essential.

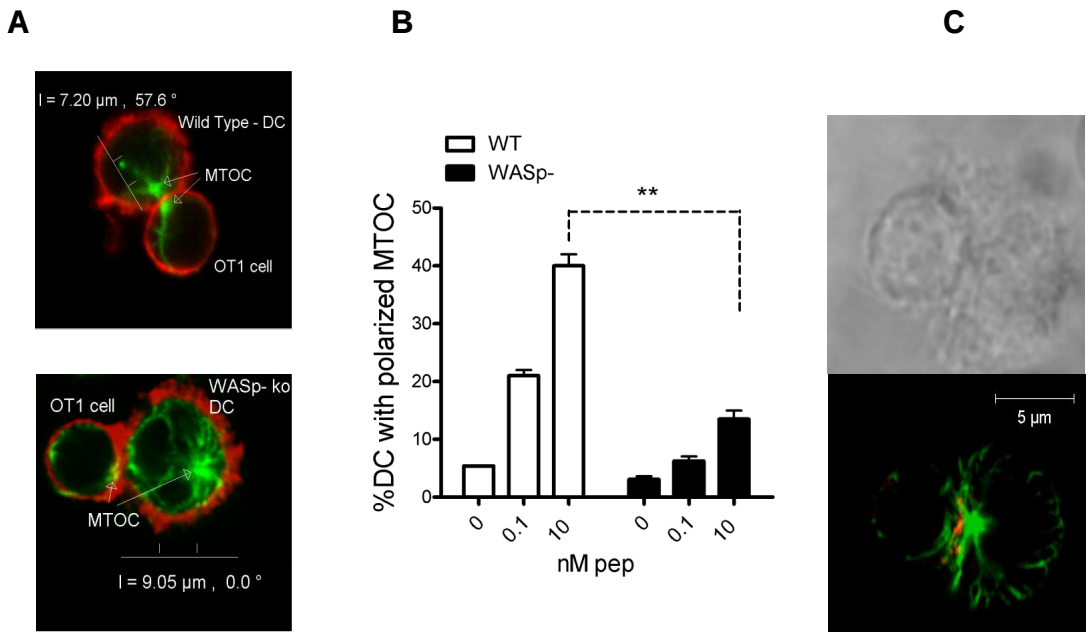


Figure 22. MTOC polarization in DCs depends on peptide dose A)BM-derived DCs of WT or WASp- mice were activated by TLR, loaded with different peptide doses, mixed with OVA specific T cells and stained with phalloidin (red) and α-tubulin (green) antibodies. Representative images of WT DCs with the MTOC polarized towards the T cell and a WASp- DC with the MTOC not reoriented. For analysis, DCs were divided in three regions and cells with the MTOC localized to the nearest region to the IS were scored as polarized. **B)** Plotted data show the percentage of DC-T conjugates with the MTOC polarized at different peptide doses. Confocal images of five independent experiments with at least 30 conjugates were analyzed for each condition. (p> 0,01). **C)** Representative confocal plane showing TCR enrichment (CD3, red) facing the polarized MTOC (α-tubulin, green) at the IS.

In order to have an indication about the kinetics of MTOC polarization we performed experiments using WT and WASp- DCs varying the times of culture with T cells. DCs were TLR stimulated, loaded with OVA-peptide (1nm). DC-T cell interactions were stopped by fixation after 15, 30 and 90 minutes. Cells were labeled with α-tubulin antibodies and analyzed by confocal microscopy. After 15 minutes we observed the highest percentage of dendritic cells polarized, this phenomenon decreased after 90 minutes of coculture. We observed a marked defect on the WASp- DCs MTOC polarization, which slightly recovered at longer interaction times. This indicates that probably the lack of the WAS protein induces a severe delay in the polarization of the MTOC towards the immune synapse (Figure 23.

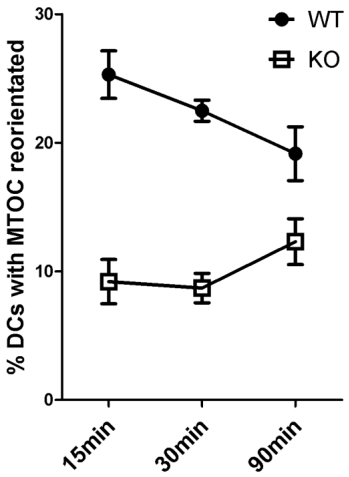


Figure 23. Kinetics of MTOC polarization. WT and WASp- DCs were TLR stimulated for 6 hours, loaded with specific OVA peptide and cocultured with naïve OT-1 cells. Interactions were stopped by fixation at indicated times. The percentage of DCs with the MTOC polarized toward the Immune synapse was quantified by confocal microscopy. Mean ± SEM of a pool of three independent experiments is plotted.

To further characterize the event of polarization in WT DCs, we evaluated the importance of maturation by TLR stimulation on cytoskeleton reorganization. It is established that TLR-stimulated, mature, DCs are more efficient in inducing T cell activation than immature DCs. We have previously shown that this correlates to formation of stronger and long-lasting DC-T cell interactions that in turn depend on an intact actin cytoskeleton[62]. We first examined the kinetics of activation of T cells and formation of conjugates after TLR stimulation. To this aim, DCs were labeled with CFSE, T cells with SNARF and mixed (1:1 ratio) at different times post TLR engagement. Green/red doublets were quantified by FACS after 20 min of interaction at 37°C. We observed that after 6hr post TLR-engagement, DCs have reached the highest capacity to cluster. This time point correlates to maximal T cell activation capacity as shown by production of IL-2 (an interleukin produced at early stages of T cell activation) in DC- T cell co-cultures (figure 24).

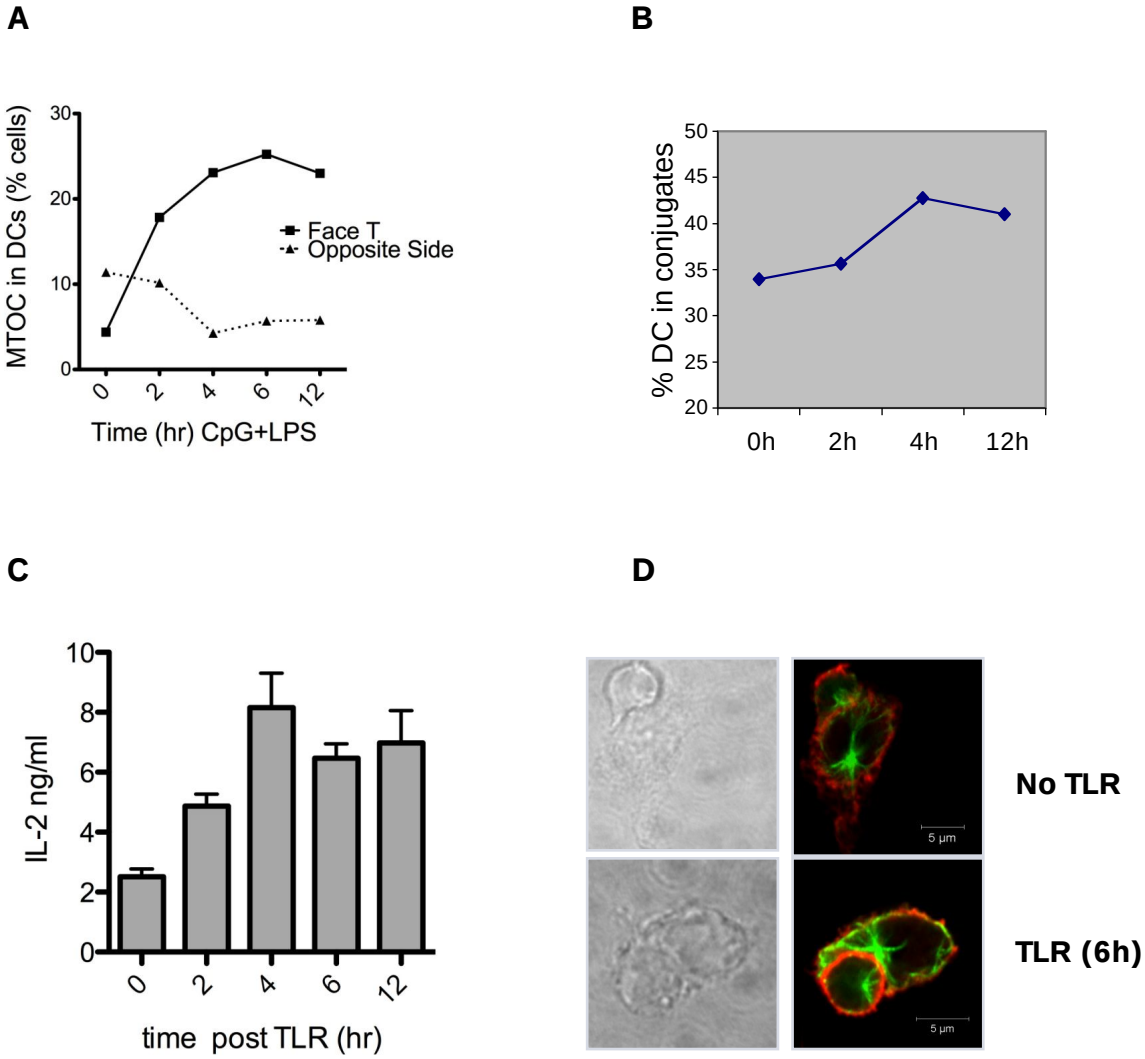


Figure 24. TLR stimulation and MTOC polarization in DCs A) DCs were treated with TLR agonist for the indicated periods and loaded with 1nM peptide before synapse formation. The percentage of polarized DCs was scored in at least 50 conjugates for each condition in three independent experiments. **B)** The formation of DC-T conjugates was assessed by FACS analysis. DC activated by TLR agonist for the indicated periods were incubated with peptide, stained with SNARF and mixed with CFSE labeled T cells. The number of Green/red doublets after 20 min of interaction was quantified for each point. Data are expressed as percentage of T cells engaged in doublets over the total number of T cells. **C)** T cell activation induced by DCs that have been stimulated by TLR agonist for the indicated periods. Peptide loaded DCs (1 nM OVA class-I peptide) were mixed to OT-I cells for 12 hr. Levels of IL-2 in the cell culture medium were measured by ELISA using standard procedures. **D)** DCs were left untreated or stimulated with a combination of TLR agonist (TLR) and loaded or not with peptide before mixing to OVA specific T cells. Cells were fixed and stained with phalloidin (red) and anti-αtubulin antibodies (green). Representative confocal images show the MTOC position for each condition.

To understand whether microtubules were preferentially polarized in mature DC-T cell contacts we cocultured T cells and antigen-loaded DCs that were or were not incubated during 2, 4, 6 and 12 hours with TLR agonists. DC-T conjugates formed by DCs that had not been stimulated by TLR agonists showed low degree of MTOC polarization. As soon as 2hr post-activation the number of conjugates with the MTOC facing the T-cell increased, reaching maximal levels at 6hr post stimulation (Figure 24). These results indicate that after 4-6 hours of TLR engagement, DCs are able to polarize the MTOC towards the IS, a time point that correlates with the maximal capacity to activate naïve T cells.

We next analyzed the dynamics of MTOC reorientation in WT DCs. To this aim we recorded by live-fluorescence imaging DCs differentiated from the BM of centrin GFP knock-in mice to allow a sharper visualization of the MTOC. Cells were pulsed with 10 nM OVA peptide, plated on coated-fibronectin chambers, mixed to labeled OT-I cells and recorded during the first 50 minutes of interaction. The MTOC spot localized mostly to a central position in isolated DCs. Upon contact with a T cell we observed DCs to traslocate the MTOC towards the membrane contacting the T cell in about half of the conjugates formed during the recording period (Figure 25 + supplementary video 7). Full MTOC polarization required 7.5±1.2 min after the initial contact. In the majority of cases (82%±5.6) once the MTOC became polarized it remained closed to the T cell membrane for the rest of the movie (up to 40 min), with little oscillation forward and back. This was valid even when the DC-T doublet moved rapidly along the x-y plane (Supplementary video 8). In few cases (9%±4) the MTOC moved on a distal position after repeated contact with the membrane facing the T cell (Figure 25b). Thus, rapidly after contact formation the DC's MTOC is repositioned in the region underneath the synaptic membrane and it remains confined in this area.

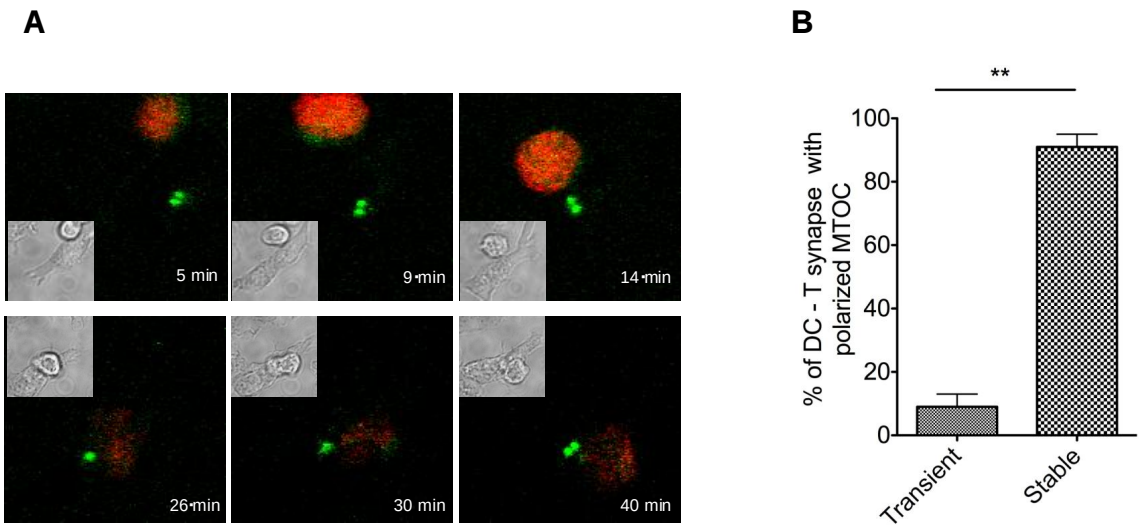


Figure 25. Time-lapse analysis of MTOC polarization. A) BM-derived centrin-gfp DCs matured by treatment with CpG/LPS were loaded with 1 nM of the MHC class-I OVA peptide and let to adhere to fibronectin. OT-I cells prestained with SNARF marker were added to the culture (1:1 ratio) and time-lapse movies were recorded during the first 50 min of interaction. Sequential images show an example of a single conjugate. In the sequence the DC forms a stable contact after few frames, then the centrin spot (green) corresponding to the MTOC, reorients towards the synapse region and remains in close proximity until the end of the video. **B)** Elapsed time for MTOC reorientation towards the contact region and duration of the MTOC polarized condition until the end of the video were measured for each T-Dc synapse. Conjugates with the MTOC polarized for at least 20 minutes were scored as stably polarized (stable) while conjugates where the MTOC was polarized and move back to the central region of the DC in less than 20 minutes were considered as transiently polarized (transient). Mean ± SEM is plotted for 60 conjugates in three independent experiments ($p<0,01$)

We next tested if MTOC polarization was exclusive of conjugates with CD8 T cells, or if it was also present in synapses formed with CD4 T lymphocytes. We performed the same assay as with OT-I cells. After TLR engagement, WT or WASp– BMDCS, were loaded or not with MHC-II OVA peptide (0,1 mg/ml) and co-cultured with CD4 naïve T cells (OT-II). After 30 minutes of interaction cells were fixed and stained with tubulin-α antibodies. We observed a very similar picture, where a high percentage of WT DCs reorient their MTOC towards the T cells in the presence of specific peptide (Figure 26). Quantification of the WASp- DCs with the MTOC polarized showed that despite little increase caused by the loading of specific peptide, there is a strong reduction with respect to WT DCs.

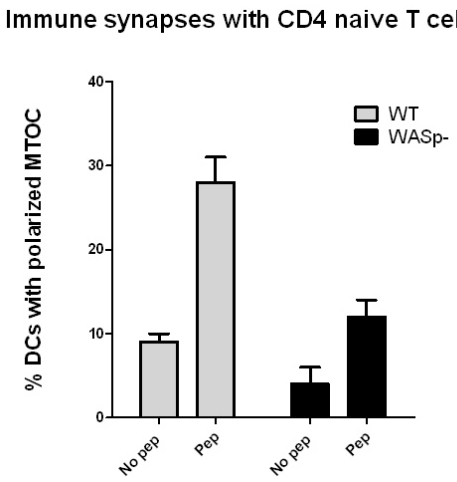


Figure 26. DCs MTOC polarization in synapses with CD4 T cells. BM-derived DCs of WT or WASp-mice were activated by TLR, loaded or not with (0,1 mg/ml) MHC II OVA peptide and mixed with OT-II T-cells. Cells were fixed and stained with phalloidin and α-tubulin antibodies and single conjugates were analyzed by confocal microscopy. The graph bar shows the percentage of DCs with the MTOC polarized towards the IS. Representative data of a series of three independent experiments was plotted (n=40).

Together these data show that DCs maturation induced by TLRs ligation confers the ability to polarize the MTOC at the IS rapidly after formation of antigen specific conjugates with T cells. The kinetic of MTOC polarization after TLR ligation corresponds to the acquisition of maximal T cell priming potential.

Role of the MTOC in DC signaling

MTOC reorientation in DCs after contact formation with T cells has not been previously reported. However, MTOC polarization is a hallmark of cell polarity in processes like cell asymmetric cell division and directional migration. In immune cells, MTOC polarization has been functionally linked to directed secretion of cytokines and lytic granules toward the target cell and most recently it was shown to be important to sustain TCR signaling. Thus, we wonder what would be the functional significance of MTOC polarization in DCs.

To this aim we first sought for intracellular localization of cytokines related with T cell activation. Interleukin 12 (IL-12), which is produced in high amounts by DCs upon TLR stimulation, has key roles in Th1/2 fate determination and in

differentiation and expansion of memory $CD8^+$ T cells. It has been suggested that IL-12 is secreted in a polarized way at the DC-NK synapse[99]. TNFα is a cytokine also produced by DCs upon TLR stimulation, has a proinflammatory role and in another cell types has been found secreted in a multidirectional way[95].

We first analyzed the kinetic of production and the intracellular distribution of these two cytokines in DCs after TLR engagement. WT BM-derived DCs were pulsed with CpG and LPS and harvested at different time points after stimulation to analyze the cytokine content in supernatants and cell lysates by ELISA. The bioactive form of IL-12 is the heterodimeric IL-12p70 composed of the p40 and the p35 chains. The IL12p40 begins to appear in the intracellular fraction as early as 1 hour post-stimulation and reaches very abundant levels by 4-6 hours before declining. Secretion and accumulation in the extracellular fraction begins at 2 hours and increases up to 18 hours post-activation (Figure27). The bioactive form IL12p70 starts to be secreted slightly later than p40 and only transiently accumulates intracellularly (2-6 hours). TNF can be detected intracellularly after 30 minutes and start to be secreted already at 1 hour, peaks at 4 hours and start to decline at 6 hours, indicating a faster secretion rate (Figure 27).

We next moved to the single cell level, to study the intracellular localization of IL-12 and TNF in DCs. WT BM-derived DCs were pulsed with CpG/LPS for different times. Cells were fixed and stained with anti-tubulin, anti -IL-12 and anti-TNF antibodies. Single cells were analyzed by confocal microscopy. A divergent intracellular staining profile between IL-12 and TNF was observed. Quantification of the distance between the cytokine containing vesicles and the MTOC revealed that at early and at late time points IL-12 is highly enriched around the MTOC. In contrast, TNF staining concentrates in a ring around the MTOC at the earliest time point (30min), but rapidly translocates through the microtubules at 1 hour and is found mostly in a MTOC distal position at later time points (4-6 hours) (Figure 28). Additionally, coimmunolabelling with TNF and IL-12 antibodies after 4 hours of TLR stimulation showed the divergent localization pattern of the two cytokines in the same cell (figure 28d).

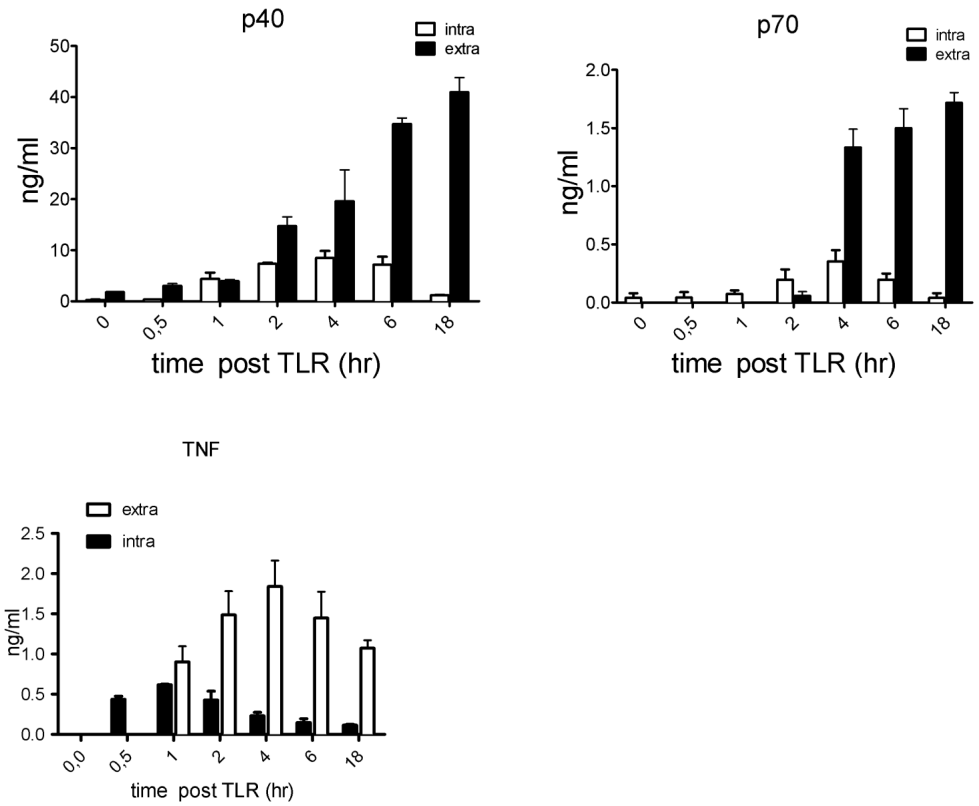


Figure 27. Kinetic of cytokines production and secretion in DCs. **A)** BM-derived DCs were stimulated with a combination of CpG and LPS for the indicated times (time post TLR). The relative content of TNF, IL-12 p40 and p70 in cell culture supernatants (black bars) and cell lysates (white bars) was determined by ELISA. Bars show means ± SEM of 5 independent experiments

In order to detect the intracellular localization of the secretory machinery in DCs, we performed immunofluorescence assays in TLR-engaged DCs, using α-tubulin and giantin (a cis-golgi marker) antibodies. We observed a closed association between the Golgi complex and the MTOC (Figure 28e).

These results indicate that IL-12 is concentrated in the MTOC/Golgi region at a time point after TLR induction that corresponds to maximal capacity to form synapses and to polarize the MTOC toward the interacting T cells. In contrast, TNF is almost completely secreted before DCs acquire full capacity to form synapses.

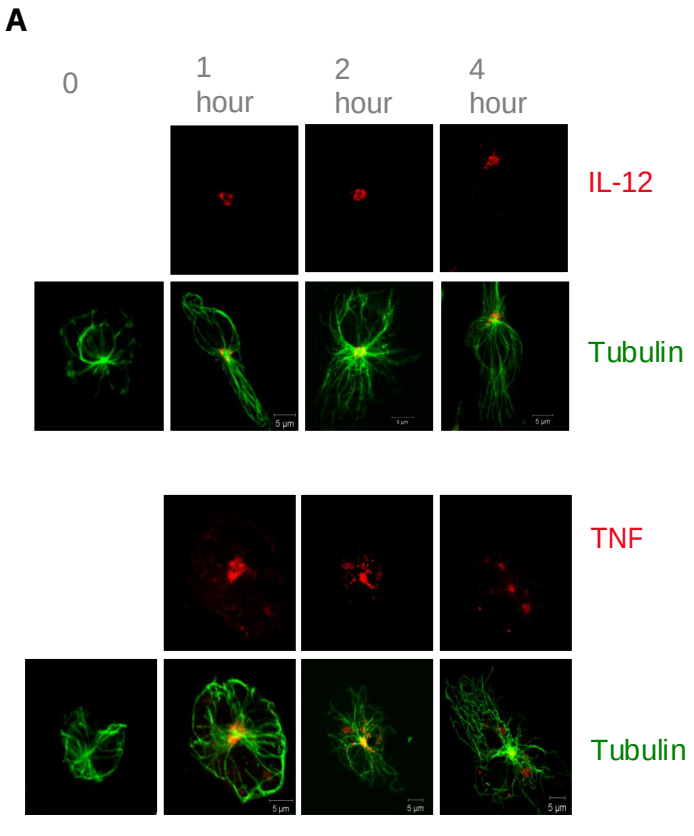

A
0
1 hour
2 hour
4 hour
IL-12
Tubulin
TNF
Tubulin
B
Relative fluorescence intensity (Tubulin)
Relative fluorescence intensity (IL-12)
IL-12
Tubulin
Diameter (px)
Relative fluorescence intensity (Tubulin)
Relative fluorescence intensity (TNF)
TNF
Tubulin
Diameter (px)
C
Average distance from MTOC (µm)
IL-12
TNF
n.s.
***
***
***
1h
2h
4h
6h
Time post TLR (hr)
D
Tubulin
IL-12
TNF
Merge
E

Figure 28. Intracellular localization of cytokines in DCs A) DCs were stimulated for different periods of time with CpG and LPS and then adhered to a fibronectin matrix and fixed. Cells were stained with alfa-tubulin (green), TNF (red) and Il-12 (red) specific antibodies. Representative confocal Z-projection images show localization of TNF and IL-12 respect to the MTOC. **B)** Histograms of the fluorescence intensity profiles of IL-12 and TNF with respect to the MTOC for one representative cell after 4 hours of TLR stimulation. **C)** DCs were stimulated with CpG/LPS at indicated times (time post TLR), fixed and immunostained. The distance (μm) from the MTOC to the IL-12 or TNF containing vesicles were measured (every point corresponds to the average of the distances for a single cell). Mean ± S.E.M was plotted for a pool of three independent experiments (n=25 DCs analyzed for each condition, P<0,001). **D)** Representative confocal images of a DC TLR stimulated for 4 hours, after fixation and co-staining with IL-12 (green), TNF (red) and α-tubulin (blue) antibodies. A contrasting localization pattern for both cytokines is observed. E) Representative confocal images of DCs TLR-stimulated for 4 hours, after fixation and co-staining with giantin (green) and alfa-tubulin (red) antibodies. Golgi apparatus is observed around the MTOC.

Next, we wanted to know if WASp deficiency affects cytokines production and intracellular localization. For WASp- DCs study we focused our attention on the analysis of IL-12 since it showed to be spatially linked to the MTOC. We first checked for the intra and extracellular levels after stimulation of the TLR performing the same assay performed for WT DCs. We observed that both fractions are considerably reduced with respect to the production of WT DCs. In particular the bioactive form p70 shows a strong reduction in the extracellular fraction (Figure 29), revealing a role of WASp in upstream events to IL-12 production.

We next moved to the single cell level analysis. WASp- DCs were TLR stimulated, fixed and immunostained. Analysis of confocal images revealed that IL-12 in WASp- DC has a distribution similar to that observed in WT DCs with the MTOC surrounded by IL-12 containing vesicles at different times of TLR stimulation (Figure 30). This suggest that absence of the WAS protein affects total IL-12 levels but does not disrupt the linkage between, Golgi, IL-12 vesicles and MTOC.

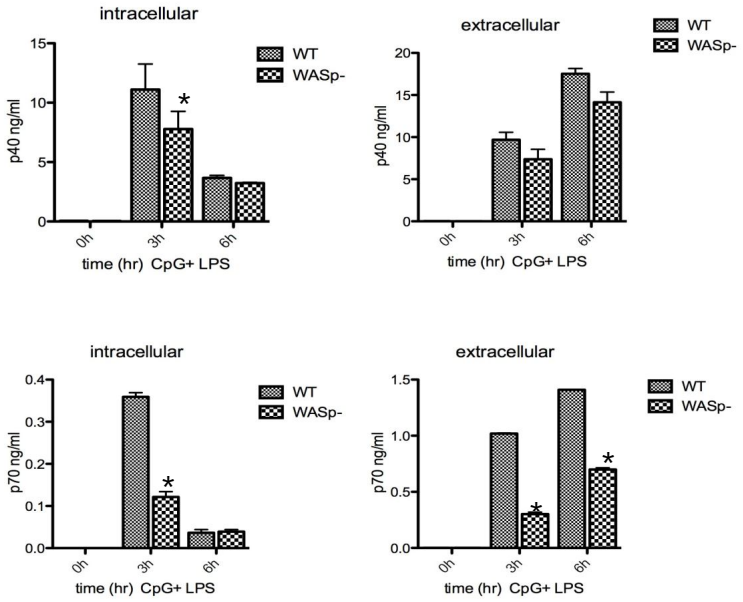


Figure 29. IL-12 production and secretion by WASp- DCs. WT and WASp- BM DCs were stimulated with a combination of CpG and LPS for the indicated times. The relative content of IL-12 p40 and p70 in cell culture supernatants (extracellular) and cell lysates (intracellular) was determined by ELISA. Data are mean values ±SEM of 3 independent experiments each with pooled cells form 3 mice/genotype. (p>0,05).

Next, we wanted to study the localization of IL-12 vesicles in the absence of microtubules. We treated DCs with a tubulin depolymerizing agent (colcemide) which prevents the linking between α and β tubulin. DCs were TLR stimulated, treated with colcemide, fixed and stained with α-tubulin and IL-12 antibodies. IL-12 is mainly found dissociated from the MTOC, both for WT and WASp- DCs treated with colcemide. This indicates that IL-12 binding to the MTOC is not WASp dependent, but it requires microtubules integrity (Figure 30).

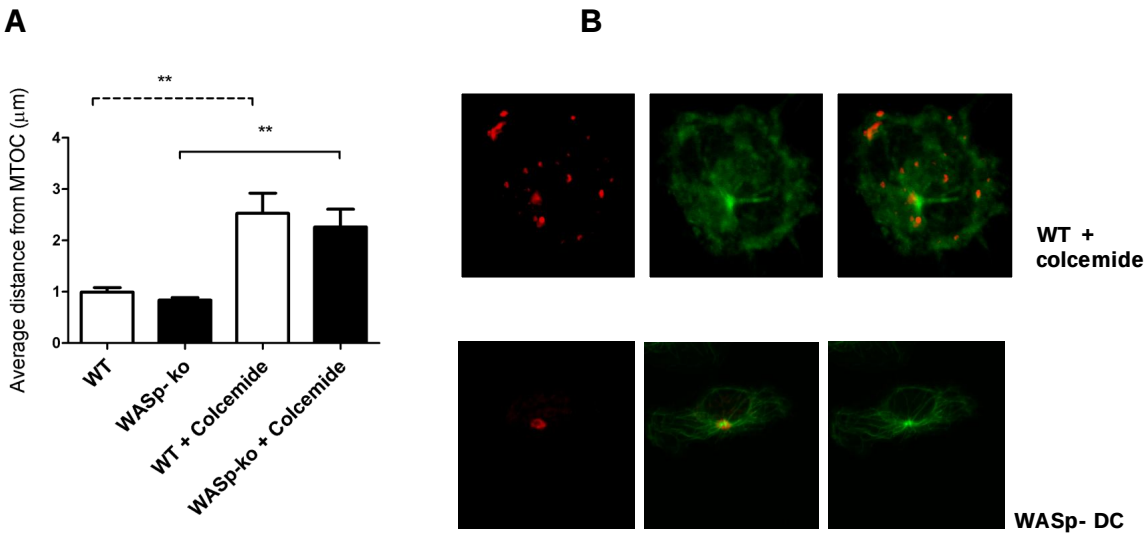


Figure 30. Association between IL-12 containing vesicles and MTOC. A) DCs were stimulated with CpG/LPS for 6 hours and treated with colcemide during the last 5 minutes of stimulation. Cells were fixed and immunolabeled with IL-12 and α -tubulin antibodies. The distance (μm) of IL-12 containing vesicles from the MTOC was measured at the single cell level. Bars represent mean values ± SEM of 25 cells/condition ($p<0.01$). B) Representative z-projection images of a colcemide treated DC (upper panel) or an untreated WASp- DC (lower panel), stained with IL-12 (red) and α-tubulin (green) antibodies.

We conclude that IL-12 is mainly found around the MTOC/Golgi region at early time points post TLR stimulation, and that this association requires microtubules integrity. The intracellular distribution of IL-12 was maintained in WASp- DCs. However the total levels of IL-12 were reduced, suggesting a role of WASp- in upstreaming events in the TLR signaling cascade.

Cytokine polarization at the immune synapse

Next, we followed the intracellular localization of cytokines during synapse formation in WT and WASp- DCs. To this aim, WT or WASp – DC were pulsed with CpG/LPS for 5hr (a time-point that correspond to the highest intracellular accumulation), loaded with increasing doses of OVA peptide, cocultured with naïve $CD8^+$ T cells during 30 minutes, fixed and immunostained for confocal analysis. To facilitate the detection of DC-T cell doublets, T cells were pre-labeled with CFSE.

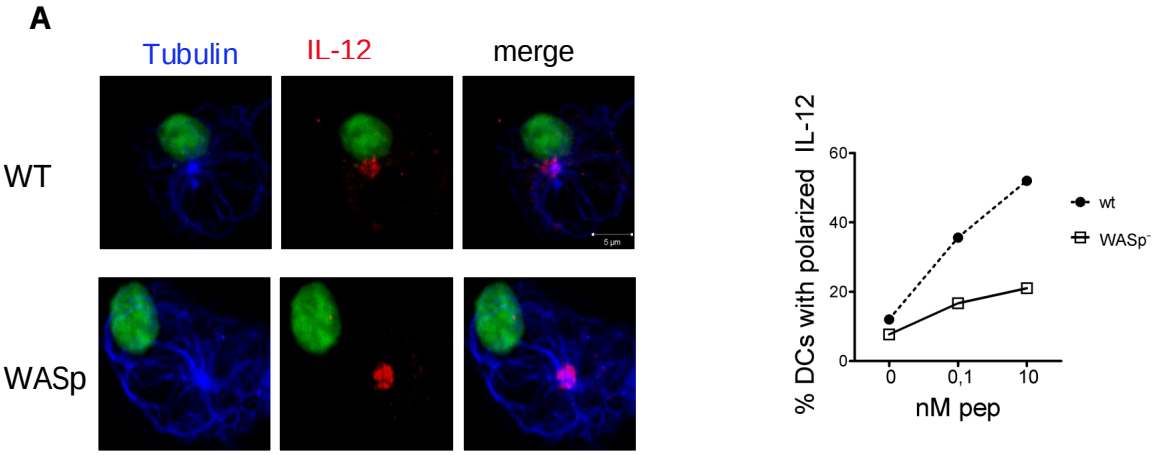
Confirming the data showed in Figure 28, labeling with anti-TNF antibodies showed that 5 hours post TLR induction only few DCs contain intracellular TNF (10-12%). Within conjugates with TNF signal in DCs, the majority of TNF was detected in vesicles close to the cell membrane with little MTOC-associated TNF at the synapse, despite TCR clustering (Figure 31d).

We quantified recruitment of IL-12 at the synapse in conjugates formed with WT DCs bearing intracellular IL-12 signal. In the presence of peptide up to 60% of conjugates showed enrichment of IL-12 at the DC-T interface. In most of the cases IL-12 enrichment correlates to TCR clustering in the T cell further confirming the requirement for TCR/MHC recognition to trigger IL-12 polarization.

Analysis of the IL-12 recruitment at the IS in WASp- cells showed a dramatic reduction (up to 80 % of reduction at the highest dose of peptide with respect to WT cells) in the percentage of WASp- DCs with polarized IL-12 vesicles (Figure 31).

To further establish a correlation between MTOC polarization and IL-12 recruitment, we used a microtubule depolymerizing drug that has been previously shown to disrupt the IL-12/MTOC association (as shown in figure 30). WT BM-derived DCs were loaded with specific OVA peptide and TLR-stimulated for 5 hours, during the last 5 minutes were treated with colchicine (equivalent to colcemide). Medium was washed away and OT-I cells were cocultured for 30 minutes, cells were fixed and stained with IL-12 and α-tubulin antibodies. Pretreatment of DCs with colchicine before synapse formation caused a significant reduction in the number of conjugates showing enriched IL-12 at the contact site (Figure 31e), without any remarkable effect on the total number of conjugates or the levels of intracellular IL-12. This data shows that IL-12 is dragged to the synapse because of its association with the MTOC.

In conclusion, these observations strongly suggest that T cells exposed to DCs in the early phases upon TLR induction receive highly concentrated IL-12. IL-12 vesicles are delivered through WASp dependent MTOC trafficking in a spatially restricted region within the synaptic cleft.



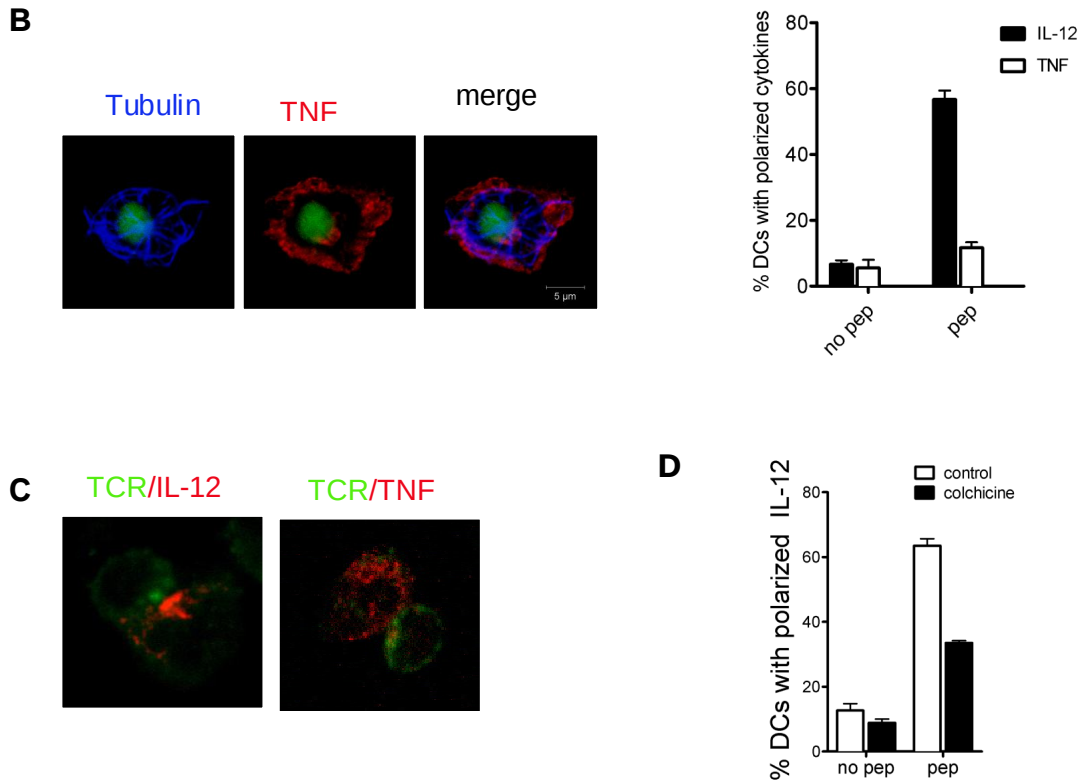


Figure 31. Cytokines polarization at the Immune Synapse. A) WT or WASp- BM-derived DC were stimulated by treatment with LPS and CpG, pulsed with increasing doses of OVA peptide, then adhered to fibronectin-coated coverslips. Cells were cocultured with OT-1 T cells previously labeled with CFSE (green) during 30 minutes, fixed and stained with α-tubulin (blue) and IL-12(red). *Left panel,* Z-projections of single DC-T conjugates showing IL-12 polarization in WT DCs opposite to WASp-. *Right panel,* single conjugates were analyzed by confocal analysis. Percentage of DCs with IL-12 polarized was quantified (see Materials). A pull of five independent experiments is plotted (n=30, for each condition) **B)** WT BM-derived DCs were TLR stimulated by treatment with LPS and CpG, pulsed or not with OVA peptide, then adhered to fibronectin-coated coverslips. Cells were cocultured with OT-1 T cells previously labeled with CFSE (green) during 30 minutes, fixed and stained with α-tubulin (blue) and TNF (red). Very few cells show a detectable TNF intracellular signal. *Left panel,* Z-projections of single DC-T conjugates showing TNF distribution in DCs, distant from the IS. *Right panel,* single conjugates were analyzed by confocal analysis. Percentage of DCs with TNF polarized was quantified (see Materials). A pull of three independent experiments is plotted (n=30, for each condition). **C)** WT BM-derived DCs TLR-stimulated and loaded with OVA peptide were mixed with OT-I cells and stained with vB5.1 (TCR, green) antibodies and IL-12 or TNF (red). Representative images of Z-plane with the highest Vβ5.1 fluorescence signal. **D)** DCs were stimulated with CpG/LPS, loaded with peptide. Cells were then rapidly treated with colchicine and mixed to OVA specific T cells. The percentage of conjugates with IL-12 enriched at the contact site was quantified. Bars represent mean values ± SEM of three independent experiments (n=50 for each condition).

We wanted to further examine cytokine polarization in WT DCs. To this aim, we immunostained DC-T cell conjugates with markers that detect coupling to the cellular membrane. We were able to visualize vesicles of IL-12 in the membrane region contacting the T cell by counterstaining the DC membrane with anti-CD11c antibodies. Furthermore, VAMP-7 positive vesicles (TI-VAMP-7 that marks the late endocytic pathway and mediates fusion of intracellular vesicles with the plasma membrane), were found enriched at the DC-T interfaces of antigen specific conjugates in close proximity to IL-12 positive vesicles. This suggests that IL-12 recruitment is accompanied by alignment of secretory organelles at the synaptic cleft (Figure 32).

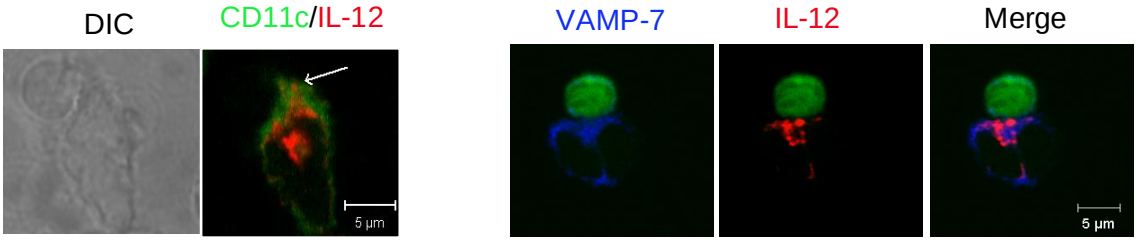


Figure 32. IL-12 localization at the IS. *Left panel*. TLR-stimulated DCs were loaded with specific peptide and mixed with OT-1 cells, stained and labelled with antibodies against CD11c(green) and IL-12(red) antibodies. One representative image of IL-12 (white arrow) distribution in the plasma membrane at the contact site is shown. *Right panel.* DC-T conjugates were formed with OT-I T cells previously stained with CFSE (green) fixed and stained with anti VAMP-7 (blue) and IL-12 (red) antibodies. A representative Z-projection of images is shown.

We finally tested our model of IL-12 polarization with different DC populations. First, we used endogenous DCs. CD11c fraction was freshly purified from the spleen of WT mice, pulsed or not with OVA peptide, TLR stimulated during 5 hours, and co-cultured with OT-I CD8+ T cells for 30 minutes. Immuonstaining with IL-12 antibodies revealed the same event of polarization towards the CD8 T cells upon antigen specific presentation (Figure 33).

We next tested if IL-12 polarization was exclusive of conjugates with CD8 T cells, or if it was also present in synapses formed with CD4 T lymphocytes. BM-derived DCs were TLR stimulated and loaded with specific OVA-MHC-II peptide, mixed with OT-II CD4 T cells, fixed and immunostained. After confocal analysis, we

observed that the percentage of DCs with IL-12 vesicles polarized greatly increased when DCs are loaded with specific peptide (Figure 33).

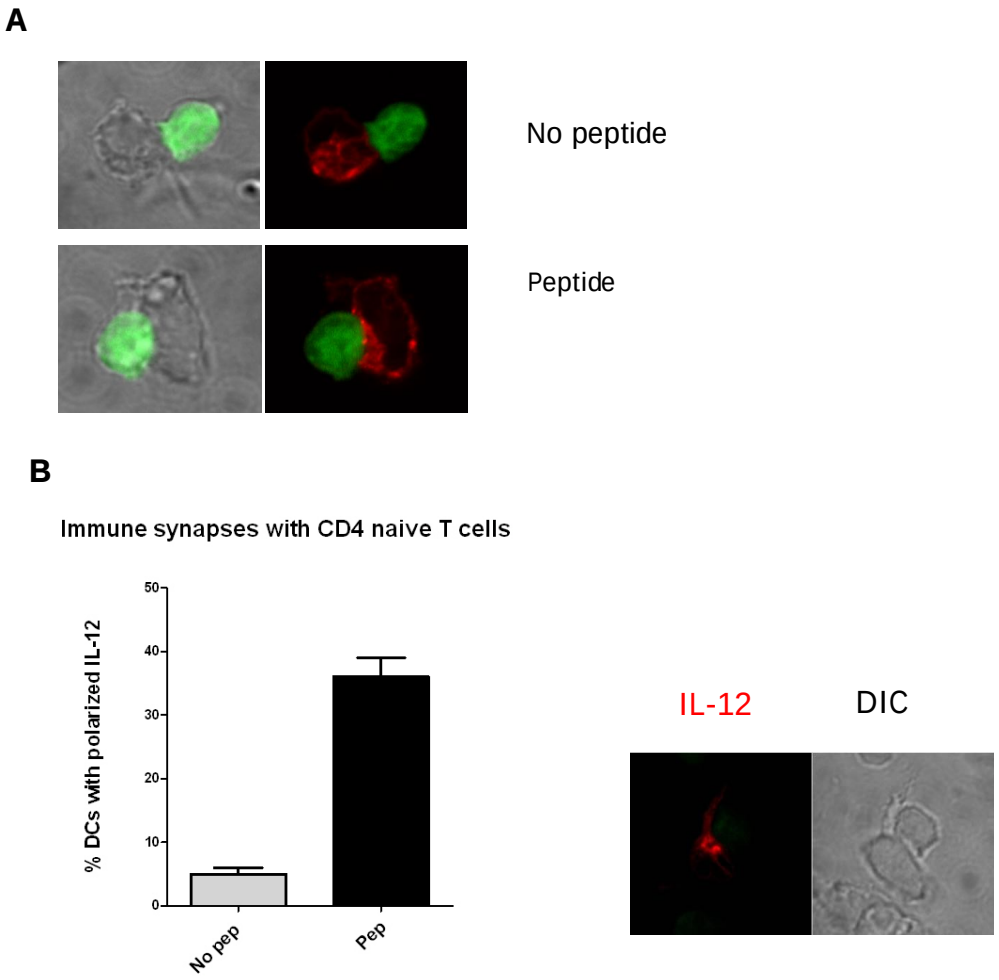


Figure 33. IL-12 polarization in DCs A) DCs from spleen of WT mice were purified. Cells were TLR stimulated, pulsed or not with OVA peptide and mixed with previously stained OT-I T cells (CFSE-green). After fixation, and immunostaining with IL-12(red) antibodies single DC-T conjugates were analyzed by confocal microscopy. Representative images of IL-12 intracellular localization in DC-T conjugates where DCs were previously loaded or not with OVA peptide are shown. **B)** BM-derived DCs were activated by TLR stimulation, loaded or not with (0,1 mg/ml) MHC II OVA peptide and mixed with OT-II Tcells. Cells were fixed and stained Il-12 (red) antibodies and single conjugates were analyzed by confocal microscopy. The graph bar shows the percentage of DCs with the IL-12 vesicles polarized towards the IS. Representative data of a series of three independent experiments was plotted (n=40). *Right panel,* a representative image of a DC-T conjugate with IL-12 concentrated at the IS region.

Therefore, early after TLR stimulation a proportion of DCs contain intracellular stores of IL-12 that are recruited to the membrane contacting the T cell during class-I and class-II antigen specific synapses formation. In contrast, TNF has already been secreted and the few remaining intracellular vesicles are mainly uncoupled form the MTOC and do not polarize at the DC-T interface.

T-cell activation after specific polarized signaling

Our results suggested that IL-12 polarization may lead to directed release toward the synaptic cleft in DC-T conjugates. Thus, we asked whether IL-12 synaptic delivery towards the IS has a functional effect on T cells.

Recently, it has been reported that cytokine receptors cluster at the DC-T interface facilitating the signaling[131]. Thus, we examined whether the IL-12 receptor is enriched at the DC-T cell synapse. BM-derived DCs were TLR stimulated and loaded with specific OVA-MHC-I peptide, mixed with OT-I T cells, fixed and immunostained with IL-12 and IL-12Rβ1 antibodies. After confocal analysis we concluded that IL-12Rβ1 gives an undetectable signal when compared to the isotype control, an event probably caused by the low levels of the receptor in naïve T cells[132] (Figure 34).

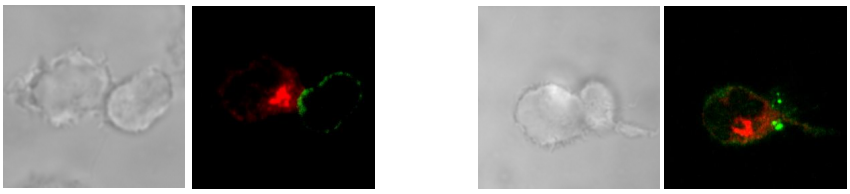


Figure 34. Failure to detect IL-12 receptor on T cells. DC-T peptide specific conjugates were stained with IL-12(red), IL-12RB1 and mouse-Isotype(green) antibodies. Pattern of staining between specific IL-12 receptor (left panel) and anti-isotype (right panel) is indistinguishable.

Nonetheless, IL-12 receptor engagement initiates a signaling cascade via the Janus-associated kinases that leads to phosphorylation of the STAT4 transcription factor and transactivation of IL-12 regulated genes[116]. Consequently, we moved to study levels of phosphorylated-STAT4 (pSTAT4) in T cells after formation of the IS.

To this aim, we set up an assay to read intracellular pSTAT4 levels by Western blot. First, OT-I cells were pulsed or not with soluble IL-12 during 30 minutes. Cells were lysed, resolved by SDS electrophoresis and blotted against pSTAT4

antibodies (see Materials). Stimulation of T cells with soluble IL-12 induced detectable levels of pSTAT4. We next studied the pSTAT4 level in T cells that had been stimulated by IL-12 bearing DCs.

BM-derived DCs were left untreated (immature) or stimulated with TLR agonists for 5hr (mature) and loaded or not with peptide. DCs were then mixed with antigen specific OT-I cells and lysed after 30' of interaction. Analysis of cell lysates by immunoblot showed a clearly detectable pSTAT4 signal upon incubation of T cells with mature DCs, but not with immature DCs, in agreement with the fact that they do not contain IL-12. Most importantly T cells mixed to mature DCs pulsed with antigen showed higher pSTAT4 levels than T cells incubated in the absence of antigen (Figure 35).

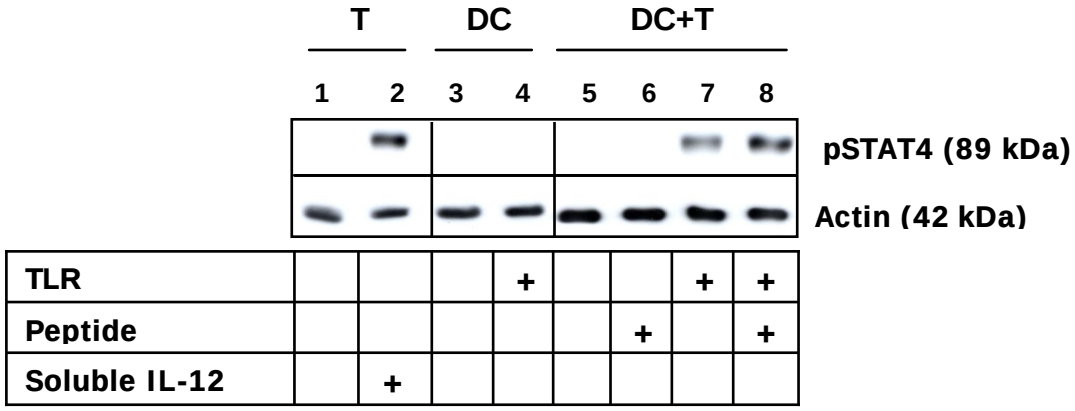
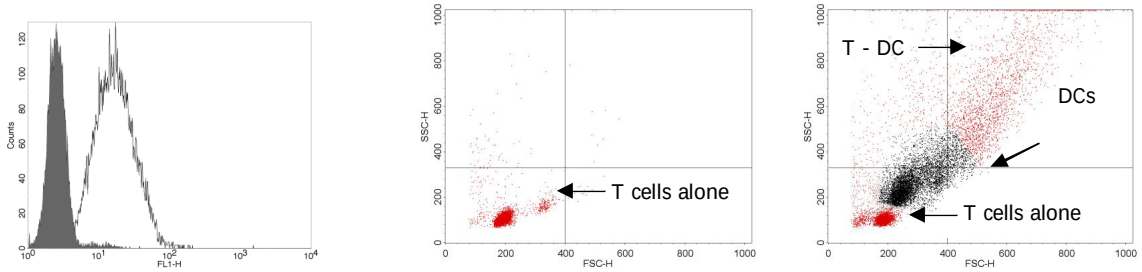


Figure 35. Activation of STAT4 in T cells upon specific antigen presentation. BM-derived DCs were pre-treated with TLRs agonist, loaded with OVA peptide and mixed with OT-I cells. After 20 minutes of incubation, cells were lysed and analyzed by Western blot against the phosphorylated form of STAT4 (pSTAT4). Control lanes (1-4) are T cells alone (1) or incubated with soluble IL-12 (2) and DCs alone not stimulated (3) or stimulated (4) with TLR agonist. Lanes 5-8: lysates of DC stimulated or not with TLR agonist (5,7) and peptide (6,8) co-incubated with T cells. A strong increase in the pSTAT4 levels is observed when T cells formed peptide specific conjugates with mature DCs.

To further analyze IL-12 signaling in T cells, we established an accurate assay to detect pSTAT4 by intracellular FACS analysis, with specific markers for DCs and T cells. OT-I cells incubated for 20' with soluble IL-12 showed a clear positive staining with anti-pSTAT4 Alexa 488 antibodies, indicating that the assay is appropriate to detect early events of phosphorylation in T cells (Figure 36). Then,

we studied pSTAT4 signaling in T cells engaged in antigen specific synapses. To this aim, DCs were left untreated (immature) or stimulated with TLR agonist for 5hr (mature), loaded or not with peptide and mixed with antigen specific OT-I cells. After 20' cells were fixed, permeabilized and labeled with anti-pSTAT4 antibodies.

The percentage of pSTAT4 positive T cells was determined by gating on the region of DC-T doublets (Figure 36). As a control, levels of pSTAT4 were also measured on T cells not engaged in synapse (see Materials). T cells alone showed a low background level similar in all cases. Instead, in DC-T doublets gates we observed a higher background and a specific pSTAT4 signal that varied depending on the DCs state. Immature DCs induced an equivalent signal regardless of the presence of peptide. In contrast, incubation with mature antigen loaded DCs as compared to not loaded DC induced an increase of 42% in the pSTAT4 signal in T cells engaged in synapse (p=0,0029) (Figure 37). Therefore, these data show that formation of antigen specific synapses increases IL-12 dependent signaling in T cells.



A **B**

Figure 36. Detection of pSTAT4 signaling by intracellular FACS analysis. A) OT-I cells were incubated in the presence of soluble IL-12 (empty curve) or control medium (gray curve) and labelled with anti-pSTAT4 Alexa-488 antibody. **B)** Dot plots of T cells alone and T cells in conjugates. *Left panel,* OT-I T cells were pulsed with IL-12 and stained with CD8 and CD45.1 markers, dot plot illustrates the FSC/SSC profile. *Right panel*, BM-DCs and OT-I cells were cocultured, fixed and stained with CD8 and CD45.1 antibodies. Double positive correspond to T cells (red dots). FSC/SSC profile illustrates the three different populations present in cocultures.

Finally we wanted to test if interfering with IL-12 polarization affected the overall phosphorylation of STAT4 in T cells in DC-T conjugates. To this aim, we use WT BM- derived DCs pretreated with colchicine and WASp- DCs. WT and WASp- BM DCs were stimulated with TLR agonists for 5hr and loaded or not with peptide. WT DCs were treated with colchicine during the last 5 minutes of incubation. DCs were mixed with antigen specific OT-I cells. After 20' cells were fixed, permeabilized and labeled with anti-pSTAT4 antibodies. In conjugates formed using colchicine-treated DCs the pSTAT4 signal was reduced of 48 % when compared to untreated DCs. Moreover, T cells exposed to WASp- DCs showed an overall lower level of pSTAT4 activation that did not increase in antigen loaded DCs synapses (Figure 37).

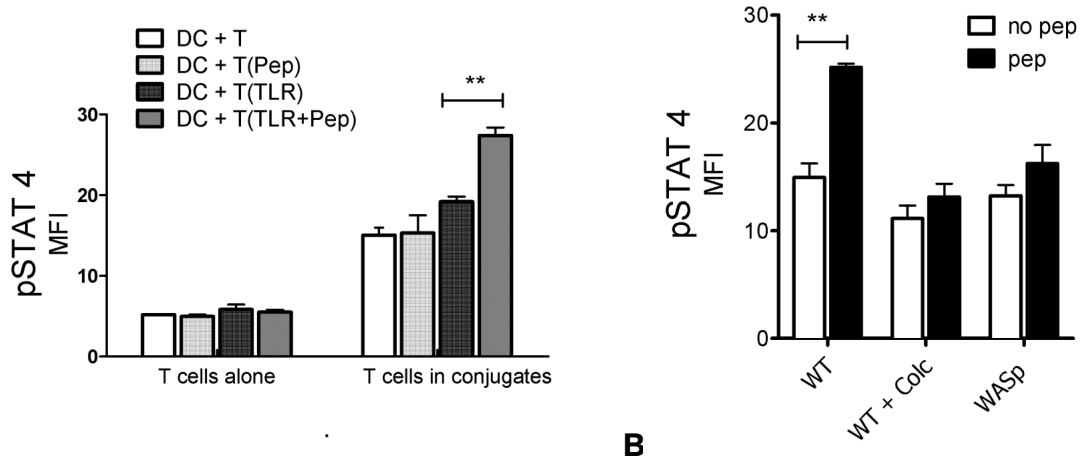


Figure 37. Detection of pSTAT4 signaling by intracellular FACS analysis. A) DCs were either resting or stimulated with TLR agonist (TLR), loaded (pep) or not loaded (no pep) with OVA peptide and mixed with OVA T-cells for 20 minutes. The median fluorescence intensity (MFI) was determined on isolated T cells (gate T cells alone) and DC-T cell doublets (gate conjugates). Data represent the mean values ± SEM (subtracted for the isotype control values), obtained in three independent experiments. **B)** Control DCs (WT), DCs treated with colchicine (colc) or WASp- DCs ($WASp^-$) were activated by TLR agonist, loaded with peptide and mixed with antigen specific T cells. Data show the MFI of pSTAT4 signal determined on T cells engaged in doublets in four independent experiments.

Together these data indicate that polarization of IL-12 vesicles in DCs is required for an enhanced IL-12 signaling in T-DC conjugates. T cells in synapse with WASp- DCs have a reduced STAT4 activation probably caused by the combined effect of a reduced MTOC polarization, a reduced IL-12 polarization toward the IS and lower levels of IL-12 secretion.

4. DISCUSSION

DCs are professional antigen-presenting cells (APC) pivotal in the initiation of primary immune responses against pathogens and in the maintenance of peripheral T-cell tolerance against self-antigens. DCs share several conserved mechanisms and pathways common to other immune cells. However, they also have unique characteristics that make them highly efficient at presenting antigens and mediating T cell responses. At present the cellular mechanisms that support the various functions they perform are not fully understood.

The actin cytoskeleton plays a main role in supporting antigen internalization and migration in DCs. A key regulator of actin polymerization in hematopoietic cells is the WAS protein. Despite clear indications of a role of WASp in the cytoarchitecture and migration of DCs[20], little is known about the effect of WASp deficiency on the ability of DCs to handle antigens and to interact with and activate naïve T-cells. Using a murine model of WASp deficiency (WASp-), here we demonstrated that WASp absence affects the capacity of DCs to internalize antigens, migrate and physically interact with T cells to support their activation.

Evaluation of the ability of DCs to uptake *Salmonella typhimurium*, revealed a severe impairment in WASp- DCs on the number of bacteria phagocytosed. After one hour of infection and using a ratio of infection 1:10 (DC-bacteria) we detected a significantly reduced number of *Salmonella* internalized by WASp- DCs. Increasing the time and ratio of infection did not correct the phagocytosis defect (data not shown). Since the *Salmonella typhimurium* strain we used was depleted for the TTSS, bacterial internalization exclusively relied on the phagocytic capacity of the DCs. Our results indicate that phagocytic capacity is strongly affected in the absence of WASp. This is in line with previous report showing that formation of the phagocytic cup is a process that relies on the polymerization and depolymerization of actin. Moreover, actin polymerization plays a key role in vesicle fission and trafficking inside the cell[133].

To study phagocytosis in DCs we performed an assay that measures the number of bacteria that were viable after internalization. Thus, to rule out that the reduced number of bacteria observed after one hour of infection was caused only by an impaired internalization in WASp- DCs and not to a higher rate of cellular processing, we proceed to analyze the intracellular compartments where the pathogen was delivered upon phagocytosis. It has been shown that *S. typhimurium* is able to manipulate the endocytic machinery to create a compartment to avoid degradation, called the salmonella containing vesicle SCV[134]. The surface markers of the SCV are still on debate, however LAMP-I a lysosomal marker related with the late endocytic pathway and EEA1- an early endosomal marker are expressed on SCV[135]. Both of them seem to be present on the compartments where *Salmonella* resides in WASp- and WT DCs. Therefore, with this bacterial model we can conclude that WASp does not affect the processing of *Salmonella typhimurium*.

In order to bypass the defective antigen internalization and to focus on the evaluation of downstream functions in further analyses of WASp- DCs, we used along our study soluble preprocessed peptides prompt to be loaded on MHC complexes.

Along with internalization and processing of particles, DCs sense pathogen associated patterns (PAMP) that are recognized by TLRs and trigger signaling cascades that enhance T cell activation. Immature DCs are mainly devoted to uptake of exogenous particles. This is supported by a high endocytic activity which is in turn regulated by the actin cytoskeleton. After TLR engagement they become mature, a process that causes an increase in the migration and in the ability to present processed antigens to activate TCR specific T cells. We observed that upregulation of maturation markers was similar in WT and WASp- DCs. However, later on when we evaluated the levels of IL-12 produced by WASp- DCs after TLR engagement by CpG and LPS, we observed a strong impairment when compared with WT DCs. The role of WASp in TLR signaling has not been studied yet,

nevertheless it has been shown in murine macrophages that stimulation of TLR4 leads to activation of an actin-Cdc42/Rac signaling pathway that specifically increase the phagocytic capacity[136]. Similarly, Arbibe et al. demonstrated that TLR2 stimulation in human cells lead to Rac activation and results in NF-kappa B transcriptional activity[137]. These studies indicate that TLR signaling and regulators of the actin/tubulin cytoskeleton may be related. Therefore, it would be relevant to examine the role of WASp in the downstream signaling of TLRs 4 and 9 in DCs.

We investigated the role of WASp in DCs ability to displace, find naïve T cells and establish stable contacts. By time-lapse video microscopy we studied the morphology and migration of DCs on a fibronectin matrix. Immature WASp- DCs displayed an altered morphology with respect to WT DCs. WASp- DCs failed to extend a polarized leading edge causing a reduction in the speed of cell migration. Maturation upon TLR engagement caused and apparent rescue of morphological abnormalities. Ruffling activity and dendrites extensions were observed equally in WT and WASp- DCs, yet WASp- cells remained slower than WT DCs. We confirmed previously reports where defective actin polymerization in WASp- DCs cause failure in the assembly of specialized substratum contacts points called podosomes and response to CCR7 gradients, resulting into defective migration[19].

We evaluated the effect of WASp- DCs impaired migration on the ability to establish contacts with T cells. By time-lapse movies we detected that WASp- DCs remained anchored to the fibronectin matrix. As a consequence, when WASp- DCs randomly contacted neighboring T cells, they were unable to trap them and to establish stable conjugates. Increasing the peptide dose presented by WASp- DCs was not enough to correct the reduced duration and frequency of contacts. These results suggested us an additional effect of WASp on synapse formation besides the reduced capacity to migrate and encounter T cells.

Our *in vivo* model confirmed data reported by Bouma et al, where BM DC WASp- adoptively transferred into wild type recipient were poor stimulators of T cell responses because of altered trafficking to secondary lymphoid organs[20]. The most plausible explanation for this behaviour in WASp- DCs is the combined effect

of defective podosome and dendrite formation and the impairment to respond to CCR7 gradients. Our study further extended on these observations by showing that not only migration to LN but also priming within LNs is affected by the absence of WASp protein in DCs. This was demonstrated by setting up the conditions to correct the homing defect of WASp- DCs to LNs and by measuring T cell activation. DCs injected in a ratio of 1:3 (WT: WASp-) resulted in an equivalent number and proper localization of WASp- DCs in LNs during the next three days. Still, when we evaluated the T cell priming upon DC encounter in LN we found that proliferation induced by WASp- DC was severely reduced. However, T cell division profiles induced by $WASp^{-}$ DCs in vivo indicates that OT-I cells do encounter antigen-bearing WASp- DCs because they enter division, but they fail to fully divide and accumulate. These data are in agreement with a recent report indicating that long-lasting stable interactions are required for full T cell expansion[138; 139].It remains to be established the long-term fate of T cells primed by WASp deficient DCs in terms of memory development.

Our study reveals a second important mechanism to explain defective priming by WASp- DCs, i.e., the inability to stabilize the interaction with T cells. So far, the analysis of the role of WASp in synapse formation was limited to its effect on T cells whereas we show here that WASp is required also on the other side of the immune synapse. WASp- cells extend ruffles similarly to WT cells indicating that WASp does not control peripheral actin protrusions in mature cells. Additional data by live imaging of cell movements in LNs proves that WASp expression in DCs is necessary to stably interact with T cells in vivo. In addition, quantification of DC-T interactions in lymph nodes evidenced reduced contact duration with $WASp^{-}$ DCs (not shown).

To further understand the impact of WASp in DCs during T cell priming we analyzed the cytoskeletal reorganization upon synapse formation with T cells. We did not notice any striking difference in the actin and tubulin cytoskeleton between WASp- and WT DCs. During this analysis we noticed that in a high proportion of WT mature DCs the MTOC was reoriented toward the region of contact with the T cells. MTOC polarization in DCs was dependent on TLR stimulation and antigen

recognition by the T cell. DCs reached the maximum capacity to reorient their MTOC after 6 hours of TLR stimulation. This may depend on the fact that mature DCs form more stable synapses and induce stronger signaling in T cells than immature DCs[62; 87]. Alternatively, intrinsic remodelling of the actin cytoskeleton induced by TLR ligation[140] may in turn affect MT dynamics enabling MTOC movements. It would be interesting to study the role of different TLRs and their effect on DCs ability to reorient the tubulin cytoskeleton.

We found that MTOC reorientation in DCs is dependent on the dose of antigen presented to the cognate T cell. This implies that surface molecules at the T cell side might trigger the signaling that leads to MTOC reorientation. In migrating astrocytes, polarization of the MTOC and Golgi is triggered by integrins that induce recruitment of cdc42 and activation of the mPar6/PKCzeta complex[141]. In T cells, LFA-1/ICAM-1 crosstalk was shown to be involved in microtubule dynamics[142]. Looking for a plausible candidate that activates tubulin cytoskeleton signaling we tried to set up and assay using latex beads coated with ICAM-1. Despite our efforts, we were not able to detect any striking difference in the reorientation of the MTOC between DCs stimulated with ICAM-1 beads or uncoated beads (not shown). This could be due to the fact that DCs recognized beads as particles to be internalized and trigger a different signaling cascade. However, it would be relevant to further study the events that lead to the polymerization of microtubules and MTOC reorientation in DCs.

MTOC reorientation is a phenomenon largely described in T cells, yet, in DCs has not been documented before. We think that this observation passed unrevealed in previous studies most likely because of the use of artificial antigen presenting cells to study events in T cells.

Immunofluorescence analysis only allows detection of the MTOC position in fixed DCs at a given time after contacting the T cell. So, it was not clear to us whether MTOC reorientation was a fluctuating or a steady event. By time-lapse videos, we established that polarization of the MTOC in DCs is a very early event occurring after contact with T cells. The reorientation of the tubulin cytoskeleton is stable

and remains even in situations where the DC-T conjugates are on movement. This indicates that MTOC polarization might play an important role in sustaining the crosstalk at the early stages of the immune synapse establishment.

MTOC polarization in DCs occurred both, during interaction with CD4 and CD8 T cells. As it has been described before, MTOC polarization is hallmark of the IS formation in T cells, NK, and macrophages[13; 68; 85; 106]. Therefore, MTOC reorientation probably makes part of a series of conserved events required to induce cell polarization towards the IS and support the informational crosstalk between interacting cells.

Our results indicate that MTOC polarization is highly reduced in WASp deficient cells. It has been demonstrated that polarization of the MTOC requires the action of the cdc42 GTPase, in T cells[103] and in NK cells it is dependent on activation-induced signaling[106]. In parallel, it has been shown that cdc42 mediates MTOC/Golgi reorientation through the Par6/aPKC pathway in fibroblasts. In order to study the pathway that leads to MTOC reorientation, we have attempted to deplete cdc42 from WT DCs and study their capacity to interact and activate T cells. Our preliminary results suggest that MTOC polarization is impaired in cdc42 null DCs (not shown). A key protein that links cdc42 activity in actin polymerization and microtubule dynamics is CIP4. CIP4 is able to interact with Cdc42 and with WASp through the SH3 domain[143] and also contains a Fes/CIP4 homology (FCH) domain, which enables it to interact with tubulin[144]. Hence, we propose that defective MTOC polarization in WASp- DCs may be related with a protein, as CIP4, that links the actin and the tubulin cytoskeleton.

The discovery of MTOC polarization occurring at the DC side upon formation of specific IS, led us to focus our attention on the description of the phenomena and its functional significance. Thus, we followed the production and secretion kinetics of IL-12 and TNF, two important cytokines in T cell activation. Both cytokines are involved on the late innate resistance and adaptive immunity responses. TNF is produced upon engagement of TLRs 2, 4 and 9 and makes part of the pro-inflammatory cytokines that control the early immune responses upon infection[97; 114; 145]. The kinetic of TNF secretion in cell cultures coupled to analysis of the

intracellular content of this cytokine indicate that is released early after TLR stimulation (2 hours) and is absent intracellularly when DCs reach a full mature phenotype (6 hours).

IL-12 has been shown to be produced in DCs upon stimulation of TLRs 4, 7 or 9. IL-12 plays a main role in the Th-1 cell differentiation and enhances the generation and cytotoxicity of T lymphocytes. The kinetic of IL-12 secretion and production in cell cultures coupled to analysis of the intracellular content of this cytokine indicated that it is produced after one hour of stimulation and reaches the highest levels of intracellular accumulation at 4-6 hours. We established a criterion that allowed us to measure the association between the cytokines and the MTOC at the single cell level. While IL-12 is found associated to the MTOC during the entire kinetics of production, TNF is produced and associated to the MTOC at the early stages and released in a multidirectional manner. Furthermore, we detected that both cytokines are produced in the Golgi, which is coupled to the MTOC.

Since we detected that the peak of IL-12 production (six hours) is synchronized with highest DCs MTOC reorientation, upregulation of costimulatory molecules and maximum ability to cluster and activate T cells, we focused our attention on the functional study of IL-12. Nonetheless, it would be interesting to study other cytokines that are produced upon TLR stimulation and might be synchronized with the acquisition of the mature phenotype. Our preliminary results indicate that IL-6 and IFN-γ are produced by DCs upon stimulation with LPS and are mainly found at the MTOC region. However, a deep study on the kinetics of production and secretion and how they correlate with intracellular stores is needed. We found that the association between the MTOC/Golgi region and the IL-12 vesicles is dependent on the integrity of the microtubules. A close association between the Golgi complex and the MTOC has been described in different cellular models. Cytoskeletal motor proteins as the dynein[146] and protein kinases such as YSK1[147] and MST4 are required to maintain the association between the MTOC and Golgi. Microtubules disruption leads to disruption of the Golgi complex. However, the remaining membrane Golgi stacks function normally, inducing glycosylation and

delivery of proteins to the cellular membrane[146]. It has been proposed that association of Golgi with the MTOC (that only occurs in animal cells) arises as an evolutionary novelty that allows them to perform directional transport and secretion. Our results are in line with this hypothesis, since DCs with disrupted microtubules, show IL-12 dispersed over the cell (Figure 30).

The Golgi complex can support microtubule nucleation[148]. Moreover, Golgi-emanating microtubules may contribute to the asymmetric microtubule networks in polarized cells and support diverse processes including post-Golgi transport to the cell front[149]. Recently it has been proposed that post translational modifications (i.e. phosphorylation, acetylation) of the tubulin may play a role, recruiting specific protein complexes and thus regulate organelle-specific properties of microtubules[150]. It would be interesting to study how the formation of antigen specific synapses regulates the association between the microtubule cytoskeleton and the Golgi.

The association between the MTOC and IL-12 vesicles led us to think that this cytokine can be transported to the IS during MTOC polarization. We provide evidences that DCs in the early phases upon TLR stimulation reorient highly concentrated IL-12 vesicles in a MTOC dependent way towards the region of contact with the interacting CD4 and CD8 T cells. Indeed, we observed a correlation between IL-12 recruitment at the IS and MTOC polarization. In WASp-DCs, that do not polarize the MTOC, IL-12 vesicles remained associated to the MTOC but were not reoriented towards the T-cell side. This picture was confirmed in WT DCs treated with tubulin-depolymerizing agents, where the association MTOC-Golgi-IL-12 was disrupted and cells did not have the ability to polarize IL-12 vesicles (Figure 30).

Lack of MTOC and cytokine polarization in WASp- DCs offers a clue to understand previous observations made in several WASp-deficient cells. WASp was shown to control via interaction with WIP and CIP4 the polarization of lytic granules in NK cells resulting in inhibition of NK cell cytotoxic activity[151; 152]. This is likely to depend on MTOC polarization although not formally demonstrated in these

studies. Similarly, our findings may provide an explanation to previous observations showing that WASp-deficient CD4 T cells are impaired to recruit and release cytokines at the IS[153].

Directed secretion has been shown to be a very useful mechanism to stimulate and communicate between different immune cells. Previous reports indicate that polarized secretion is always accompanied by translocation of the MTOC and the secretory machinery. Cytokine secretion by immune cells can be either constitutive or regulated through interaction of vesicles with the plasma membrane and Ca^{2+} membrane fusion. The physical contact between vesicles and the plasma membrane is mediated by the N-ethlymalemide-sensitive factor attachment protein receptors (SNARE). The classification of the SNARE is still under discussion[154]. According to the classical model, vesicle-associated ligands (v-SNARE) specifically recognize and interact with receptor target molecules (t-SNAREs) found in the plasma membrane to form SNARE complexes which mediate membrane fusion and exocytosis. Various studies have been focused on the role of SNARE proteins in the immune system. Das et al. show that the t-SNAREs cluster at the immunological synapse of Jurkat T cells. Also, inactivation of the v-SNARES VAMP2 and VAMP3 impairs the delivery and accumulation of TCRs at the immunological synapse[113]. The v-SNARE protein Ti-VAMP-7 is present both in the Golgi apparatus and in the endosomal system and has been involved in inflammatory responses mediated by mastocytes, basophils, eosinophils and macrohages[154]. TI-VAMP-7 is also involved in the secretion of lysosomal granules by NK cells. We found that IL-12 vesicles are beneath the cell membrane in the synapse region surrounded by VAMP7, suggesting that IL-12 release is mediated by a SNARE complex in a polarized way toward the interacting T cell.

It has been proposed that WASp play a key role in the redistribution of SNAREs to the IS in T cells. Morales-Tirado et al. have shown that WASp absence in T cells is required for the polarization of cytokines toward antigen presenting cells and blocks the secretory pathway of IL-2, IL-4 and IFN-g, but does not disrupt chemokines trafficking[153]. It would be interesting to study in details the role of

WASp in the trafficking of the SNARE complex and its role in the directed secretion of cytokines in DCs.

The finding that DCs polarize IL-12 during antigen presentation is interesting in terms of the potential role in T cell programming. Antigen presentation by immature DCs to naïve CD8+ T cells results in tolerance induction, whereas presentation by mature DCs stimulates the generation of CTLs and memory[155; 156; 157]. The increased capacity to present specific antigens and to upregulate costimulatory signals is considered the major basis for T cell activation by mature DCs. However, it has been demonstrated that T cell activation by antigen loaded DCs expressing high levels of B7 can fail[158]. Hochrein et al demonstrated that artificial antigen presenting cells (aAPC), which only deliver antigen and B7 signals, are effective in stimulating clonal expansion and development of cytolytic activity by memory cells but are ineffective in stimulating responses by naïve cells. However, when IL-12 was added to the culture medium, a strong clonal expansion and development of cytolytic activity by naïve T cells was observed [159]. These experiments demonstrated that IL-12 must be delivered as a third signal, in order to induce an effective immune response[160].

We tried different strategies to prove that IL-12 is secreted in a polarized way and induces signaling events on T cells. We first tried to detect the localization of the IL-12 receptor in T cells upon stimulation with IL-12. It has been previously reported that naïve T cells express very low levels of the IL-12β2 receptor, and coupling of IL-12 induces a positive loop of expression and delivery to the membrane. We failed to track the recruitment of the IL-12 receptor probably because we checked at very early time points where the activation events had not occur yet and the IL-12 receptor is found at basal levels. However, it has been described that IL-12Rβ2 expressed at low levels in naive OT-I T cells is able to signal and impact the response to antigen stimulation[132].

As a second approach we studied the downstream events of IL-12 receptor engagement. The specific cellular effects of IL-12 are mainly mediated by phosphorylation of the transcription factor STAT4. It has been shown that IL-12

exposure during antigen stimulation induces transient phosphorylation of STAT4 which conditions CD8 cells for robust effector differentiation and long-term responses[132]. Our experimental models, allowed us to detect phosphorylation of STAT 4 in T cells briefly after stimulation with soluble IL-12. The absence of pSTAT4 in synapses with immature DCs confirmed that IL-12 produced upon TLR stimulation is required to induce STAT4 signaling in T cells. Culture of T cells with mature DCs in absence of antigen induced detectable levels of pSTAT4 in T cells found in conjugates. More interesting, synapses formed with mature DCs bearing a specific antigen induce higher levels of pSTAT4 in T cells. These data strongly suggest that IL-12 is delivered through the synapse in antigen-specific DC-T cell conjugates, leading to stronger T cell activation that exposure to soluble IL-12.

To correlate induction of pSTAT4 signaling to MTOC reorientation we used as a model DCs that based on our results were unable to polarize the MTOC (Figure 33). In the case of colchicine treated DCs (association IL-12 and microtubules disrupted / IL-12 not polarized at the IS) we detected reduced levels of STAT4 phosphorylation in T cells forming conjugates, regardless the peptide dose. In the case of WASp- DCs (IL-12 and MTOC associated / IL-12 not polarized at the IS) we observed a similar picture with a significantly lower concentration of pSTAT4 in T cells with respect to conjugates formed with WT DCs. Unfortunately, a clear interpretation of the data obtained with WASp- DCs is hampered by the reduced levels of IL-12 produced by WASp- cells (Figure 29). Therefore, we conclude that the decreased levels of STAT4 signaling induced by WASp- DCs are caused by the combined deficiency in IL-12 production upon TLR stimulation and defective MTOC/Golgi polarization towards the IS.

Despite a lot of information on the pathways that induce IL-12 production in DCs and on its effects on T-cell differentiation[116; 161], this is to our knowledge the first evidence that IL-12 signals can be transmitted synaptically to T cells during priming, thus enhancing the local concentration that is delivered to antigen specific T cells. Based on our data, synaptic transmission would be limited to a short period of time in the DCs maturation program, i.e., early after TLR ligation (4-6hr), when DCs contain abundant intracellular IL-12. Importantly, at this time

point DCs have processed and transported to the membrane pathogen derived antigens and they have acquired full capacity to interact with T cells. At later time points fully mature DCs are devoid of intracellular IL-12, suggesting that interaction with T cells at this stage would not result in a strong burst in IL-12 signaling transmitted through the synapse. It is interesting to speculate that this would have important implications for T-cell fate determination.

Here, we revealed a mechanism for DCs polarization in IS that enhances the signaling towards the interacting T cell. Studies in different cellular models have shown that polarity is controlled by five interrelated systems[73]. Besides microtubule cytoskeleton and vesicle trafficking, the actomyosin cytoskeleton, surface receptors and polarity proteins contribute to establish and modulate cell architecture. It would be interesting to study the role of each of these systems in the polarization of DCs.

Altogether, this study discloses new important mechanisms to understand WAS pathogenesis. The inability of WASp- DCs to properly polarize the MTOC at the IS may affect focal delivery of adhesion molecules thus explaining reduced DC-T contact duration. In addition defective MTOC polarization reduces polarization of soluble mediators like IL-12 that combined with an impaired IL-12 production upon TLR signaling certainly contributes to the strong defect observed on T-cell priming.

5. CONCLUSIONS

In this work we demonstrate that WASp has a key role at different levels of DC functioning and it is required to properly activate T cells. This is the first report that demonstrates a direct effect of WASp on synapse formation and T cell activation by DCs beyond their role on cell migration.

By studying the role of WASp in DCs we discovered that mature DCs polarize their tubulin cytoskeleton towards the interacting T cell during antigen recognition. We show that the capacity of DCs to polarize the MTOC is synchronized with the acquisition of a mature phenotype upon TLR engagement that correlates with the maximum ability to form conjugates and activate T cells. Moreover, we demonstrate that WASp- DCs have both, a defective TLR signaling and an impairment to reorient the MTOC.

Evaluating the functional implication of polarization in DCs we demonstrate that the MTOC/Golgi/IL-12 complex is relocated beneath the IS region upon formation of antigen-specific synapses. Remarkably, IL-12 signals can be transmitted synaptically to T cells during priming, thus enhancing the local concentration that is delivered to antigen-specific T cells, inducing a stronger downstream signaling. The directed delivery of IL-12 is dependent on MTOC reorientation, thus WASp-DCs provoke an ineffective response in T cells.

Altogether, our data are of interest for the understanding of DCs biology and its role in T-cell activation and provide the basis for new studies on the polarization events in DCs upon synapse formation. Besides basic knowledge, this study provides interesting clues to understand the cellular basis of WAS pathogenesis.

6. BIBLIOGRAPHY

1. Machesky, L. M. & Gould, K. L. (1999). The Arp2/3 complex: a multifunctional actin organizer. *Curr Opin Cell Biol* **11**, 117-21.
2. Paunola, E., Mattila, P. K. & Lappalainen, P. (2002). WH2 domain: a small, versatile adapter for actin monomers. *FEBS Lett* **513**, 92-7.
3. Martinez-Quiles, N., Rohatgi, R., Anton, I. M., Medina, M., Saville, S. P., Miki, H., Yamaguchi, H., Takenawa, T., Hartwig, J. H., Geha, R. S. & Ramesh, N. (2001). WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat Cell Biol* **3**, 484-91.
4. Higgs, H. N. & Pollard, T. D. (2000). Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J Cell Biol* **150**, 1311-20.
5. Snapper, S. B. & Rosen, F. S. (1999). The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization. *Annu Rev Immunol* **17**, 905-29.
6. Badour, K., Zhang, J., Shi, F., McGavin, M. K., Rampersad, V., Hardy, L. A., Field, D. & Siminovitch, K. A. (2003). The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. *Immunity* **18**, 141-54.
7. Thrasher, A. J. (2002). WASp in immune-system organization and function. *Nat Rev Immunol* **2**, 635-46.
8. Lorenzi, R., Brickell, P. M., Katz, D. R., Kinnon, C. & Thrasher, A. J. (2000). Wiskott-Aldrich syndrome protein is necessary for efficient IgG-mediated phagocytosis. *Blood* **95**, 2943-6.
9. Orange, J. S., Ramesh, N., Remold-O'Donnell, E., Sasahara, Y., Koopman, L., Byrne, M., Bonilla, F. A., Rosen, F. S., Geha, R. S. & Strominger, J. L. (2002). Wiskott-Aldrich syndrome protein is required for NK cell cytotoxicity and colocalizes with actin to NK cell-activating immunologic synapses. *Proc Natl Acad Sci U S A* **99**, 11351-6.
10. Westerberg, L., Larsson, M., Hardy, S. J., Fernandez, C., Thrasher, A. J. & Severinson, E. (2005). Wiskott-Aldrich syndrome protein deficiency leads to reduced B-cell adhesion, migration, and homing, and a delayed humoral immune response. *Blood* **105**, 1144-52.
11. Zhang, J., Shehabeldin, A., da Cruz, L. A., Butler, J., Somani, A. K., McGavin, M., Kozieradzki, I., dos Santos, A. O., Nagy, A., Grinstein, S., Penninger, J. M. & Siminovitch, K. A. (1999). Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J Exp Med* **190**, 1329-42.
12. Zhang, J., Shi, F., Badour, K., Deng, Y., McGavin, M. K. & Siminovitch, K. A. (2002). WASp verprolin homology, cofilin homology, and acidic region domain-mediated actin polymerization is required for T cell development. *Proc Natl Acad Sci U S A* **99**, 2240-5.
13. Linder, S., Higgs, H., Hufner, K., Schwarz, K., Pannicke, U. & Aepfelbacher, M. (2000). The polarization defect of Wiskott-Aldrich syndrome macrophages is linked to dislocalization of the Arp2/3 complex. *J Immunol* **165**, 221-5.

14. Sasahara, Y., Rachid, R., Byrne, M. J., de la Fuente, M. A., Abraham, R. T., Ramesh, N. & Geha, R. S. (2002). Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol Cell* **10**, 1269-81.
15. Westerberg, L., Greicius, G., Snapper, S. B., Aspenstrom, P. & Severinson, E. (2001). Cdc42, Rac1, and the Wiskott-Aldrich syndrome protein are involved in the cytoskeletal regulation of B lymphocytes. *Blood* **98**, 1086-94.
16. Snapper, S. B., Rosen, F. S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C. H., Hagemann, T. L., Kwan, S. P., Ferrini, R., Davidson, L., Bhan, A. K. & Alt, F. W. (1998). Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* **9**, 81-91.
17. Cotta-de-Almeida, V., Westerberg, L., Maillard, M. H., Onaldi, D., Wachtel, H., Meelu, P., Chung, U. I., Xavier, R., Alt, F. W. & Snapper, S. B. (2007). Wiskott Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. *Proc Natl Acad Sci U S A* **104**, 15424-9.
18. Binks, M., Jones, G. E., Brickell, P. M., Kinnon, C., Katz, D. R. & Thrasher, A. J. (1998). Intrinsic dendritic cell abnormalities in Wiskott-Aldrich syndrome. *Eur J Immunol* **28**, 3259-67.
19. de Noronha, S., Hardy, S., Sinclair, J., Blundell, M. P., Strid, J., Schulz, O., Zwirner, J., Jones, G. E., Katz, D. R., Kinnon, C. & Thrasher, A. J. (2005). Impaired dendritic-cell homing in vivo in the absence of Wiskott-Aldrich syndrome protein. *Blood* **105**, 1590-7.
20. Bouma, G., Burns, S. & Thrasher, A. J. (2007). Impaired T-cell priming in vivo resulting from dysfunction of WASp-deficient dendritic cells. *Blood* **110**, 4278-84.
21. Merad, M. & Manz, M. G. (2009). Dendritic cell homeostasis. *Blood* **113**, 3418-27.
22. Gallegos, A. M. & Bevan, M. J. (2006). Central tolerance: good but imperfect. *Immunol Rev* **209**, 290-6.
23. Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. [Review]. *Annual Review of Immunology* **9**, 271-96.
24. Ginhoux, F., Collin, M. P., Bogunovic, M., Abel, M., Leboeuf, M., Helft, J., Ochando, J., Kissenpfennig, A., Malissen, B., Grisotto, M., Snoeck, H., Randolph, G. & Merad, M. (2007). Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp Med* **204**, 3133-46.
25. Steinman, R. M. & Witmer, M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U S A* **75**, 5132-6.
26. Randolph, G. J., Ochando, J. & Partida-Sanchez, S. (2008). Migration of dendritic cell subsets and their precursors. *Annu Rev Immunol* **26**, 293-316.
27. Shortman, K. & Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. *Nat Rev Immunol* **2**, 151-61.
28. den Haan, J. M., Lehar, S. M. & Bevan, M. J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* **192**, 1685-96.
29. Colonna, M., Trinchieri, G. & Liu, Y. J. (2004). Plasmacytoid dendritic cells in immunity. *Nat Immunol* **5**, 1219-26.

30. Nakano, H., Yanagita, M. & Gunn, M. D. (2001). CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* **194**, 1171-8.
31. Dai, J., Megjugorac, N. J., Amrute, S. B. & Fitzgerald-Bocarsly, P. (2004). Regulation of IFN regulatory factor-7 and IFN-alpha production by enveloped virus and lipopolysaccharide in human plasmacytoid dendritic cells. *J Immunol* **173**, 1535-48.
32. Shortman, K. & Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* **7**, 19-30.
33. Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K. & Wu, L. (2005). Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J Immunol* **174**, 6592-7.
34. Garrett, W. S., Chen, L. M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J. E. & Mellman, I. (2000). Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* **102**, 325-34.
35. Trombetta, E. S. & Mellman, I. (2005). Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol* **23**, 975-1028.
36. Banchereau, J. & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* **392**, 245-52.
37. Conner, S. D. & Schmid, S. L. (2003). Regulated portals of entry into the cell. *Nature* **422**, 37-44.
38. Symons, M. & Rusk, N. (2003). Control of vesicular trafficking by Rho GTPases. *Curr Biol* **13**, R409-18.
39. Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. & Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* **20**, 621-67.
40. Schulz, O. & Reis e Sousa, C. (2002). Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology* **107**, 183-9.
41. Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M. & Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science* **315**, 107-11.
42. Vigneron, N., Stroobant, V., Chapiro, J., Ooms, A., Degiovanni, G., Morel, S., van der Bruggen, P., Boon, T. & Van den Eynde, B. J. (2004). An antigenic peptide produced by peptide splicing in the proteasome. *Science* **304**, 587-90.
43. Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**, 1283-8.
44. Delamarre, L., Pack, M., Chang, H., Mellman, I. & Trombetta, E. S. (2005). Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* **307**, 1630-4.
45. Heath, W. R. & Carbone, F. R. (2001). Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol* **19**, 47-64.

46. Schmid, D., Pypaert, M. & Munz, C. (2007). Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* **26**, 79-92.
47. Villadangos, J. A. & Schnorrer, P. (2007). Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol* **7**, 543-55.
48. Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* **54 Pt 1**, 1-13.
49. Iwasaki, A. & Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nat Immunol* **5**, 987-95.
50. Kawai, T. & Akira, S. (2006). Innate immune recognition of viral infection. *Nat Immunol* **7**, 131-7.
51. MartIn-Fontecha, A., Sebastiani, S., Hopken, U. E., Uguccioni, M., Lipp, M., Lanzavecchia, A. & Sallusto, F. (2003). Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* **198**, 615-21.
52. Bajenoff, M., Granjeaud, S. & Guerder, S. (2003). The strategy of T cell antigen-presenting cell encounter in antigen-draining lymph nodes revealed by imaging of initial T cell activation. *J Exp Med* **198**, 715-24.
53. Vassileva, G., Soto, H., Zlotnik, A., Nakano, H., Kakiuchi, T., Hedrick, J. A. & Lira, S. A. (1999). The reduced expression of 6Ckine in the plt mouse results from the deletion of one of two 6Ckine genes. *J Exp Med* **190**, 1183-8.
54. Kerksiek, K. M., Niedergang, F., Chavrier, P., Busch, D. H. & Brocker, T. (2005). Selective Rac1 inhibition in dendritic cells diminishes apoptotic cell uptake and cross-presentation in vivo. *Blood* **105**, 742-9.
55. Benvenuti, F., Hugues, S., Walmsley, M., Ruf, S., Fetler, L., Popoff, M., Tybulewicz, V. L. & Amigorena, S. (2004). Requirement of Rac1 and Rac2 expression by mature dendritic cells for T cell priming. *Science* **305**, 1150-3.
56. Veeraswamy, R. K., Cella, M., Colonna, M. & Unanue, E. R. (2003). Dendritic cells process and present antigens across a range of maturation states. *J Immunol* **170**, 5367-72.
57. Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. J. & Ploegh, H. L. (2000). Viral subversion of the immune system. *Annu Rev Immunol* **18**, 861-926.
58. Allan, R. S., Waithman, J., Bedoui, S., Jones, C. M., Villadangos, J. A., Zhan, Y., Lew, A. M., Shortman, K., Heath, W. R. & Carbone, F. R. (2006). Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* **25**, 153-62.
59. Belz, G. T., Smith, C. M., Kleinert, L., Reading, P., Brooks, A., Shortman, K., Carbone, F. R. & Heath, W. R. (2004). Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci U S A* **101**, 8670-5.
60. Leon, B., Lopez-Bravo, M. & Ardavin, C. (2007). Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* **26**, 519-31.

61. Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M. & Nussenzweig, M. C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* **194**, 769-79.
62. Benvenuti, F., Lagaudriere-Gesbert, C., Grandjean, I., Jancic, C., Hivroz, C., Trautmann, A., Lantz, O. & Amigorena, S. (2004). Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes. *J Immunol* **172**, 292-301.
63. Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. & Dustin, M. L. (1999). The immunological synapse: a molecular machine controlling T cell activation [see comments]. *Science* **285**, 221-7.
64. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82-6.
65. Cemerski, S., Das, J., Giurisato, E., Markiewicz, M. A., Allen, P. M., Chakraborty, A. K. & Shaw, A. S. (2008). The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. *Immunity* **29**, 414-22.
66. Lee, K. H., Holdorf, A. D., Dustin, M. L., Chan, A. C., Allen, P. M. & Shaw, A. S. (2002). T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539-42.
67. Vicente-Manzanares, M. & Sanchez-Madrid, F. (2004). Role of the cytoskeleton during leukocyte responses. *Nat Rev Immunol* **4**, 110-22.
68. Stinchcombe, J. C., Majorovits, E., Bossi, G., Fuller, S. & Griffiths, G. M. (2006). Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* **443**, 462-5.
69. Zeng, R., Cannon, J. L., Abraham, R. T., Way, M., Billadeau, D. D., Bubeck-Wardenberg, J. & Burkhardt, J. K. (2003). SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *J Immunol* **171**, 1360-8.
70. Cannon, J. L. & Burkhardt, J. K. (2004). Differential roles for Wiskott-Aldrich syndrome protein in immune synapse formation and IL-2 production. *J Immunol* **173**, 1658-62.
71. Wulfing, C. & Davis, M. M. (1998). A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* **282**, 2266-9.
72. Wulfing, C., Sjaastad, M. D. & Davis, M. M. (1998). Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. *Proc Natl Acad Sci U S A* **95**, 6302-7.
73. Krummel, M. F. & Macara, I. (2006). Maintenance and modulation of T cell polarity. *Nat Immunol* **7**, 1143-9.
74. Xavier, R. & Seed, B. (2005). PDZ domains and the politics of polarity in lymphocytes. *Immunity* **22**, 655-6.
75. Stinchcombe, J. C., Bossi, G., Booth, S. & Griffiths, G. M. (2001). The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751-61.
76. Luders, J. & Stearns, T. (2007). Microtubule-organizing centres: a re-evaluation. *Nat Rev Mol Cell Biol* **8**, 161-7.

77. Combs, J., Kim, S. J., Tan, S., Ligon, L. A., Holzbaur, E. L., Kuhn, J. & Poenie, M. (2006). Recruitment of dynein to the Jurkat immunological synapse. *Proc Natl Acad Sci U S A* **103**, 14883-8.
78. Billadeau, D. D., Nolz, J. C. & Gomez, T. S. (2007). Regulation of T-cell activation by the cytoskeleton. *Nat Rev Immunol* **7**, 131-43.
79. Viola, A., Contento, R. L. & Molon, B. (2006). T cells and their partners: The chemokine dating agency. *Trends Immunol* **27**, 421-7.
80. Bousso, P. & Robey, E. (2003). Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol* **4**, 579-85.
81. Miller, M. J., Wei, S. H., Parker, I. & Cahalan, M. D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* **296**, 1869-73.
82. Mempel, T. R., Henrickson, S. E. & Von Andrian, U. H. (2004). T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* **427**, 154-9.
83. Molon, B., Gri, G., Bettella, M., Gomez-Mouton, C., Lanzavecchia, A., Martinez, A. C., Manes, S. & Viola, A. (2005). T cell costimulation by chemokine receptors. *Nat Immunol* **6**, 465-71.
84. Al-Alwan, M. M., Rowden, G., Lee, T. D. & West, K. A. (2001). Fascin is involved in the antigen presentation activity of mature dendritic cells. *J Immunol* **166**, 338-45.
85. Al-Alwan, M. M., Liwski, R. S., Haeryfar, S. M., Baldridge, W. H., Hoskin, D. W., Rowden, G. & West, K. A. (2003). Cutting edge: dendritic cell actin cytoskeletal polarization during immunological synapse formation is highly antigen-dependent. *J Immunol* **171**, 4479-83.
86. Kondo, T., Cortese, I., Markovic-Plese, S., Wandinger, K. P., Carter, C., Brown, M., Leitman, S. & Martin, R. (2001). Dendritic cells signal T cells in the absence of exogenous antigen. *Nat Immunol* **2**, 932-8.
87. Hugues, S., Fetler, L., Bonifaz, L., Helft, J., Amblard, F. & Amigorena, S. (2004). Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol* **5**, 1235-42.
88. Bloom, O., Unternaehrer, J. J., Jiang, A., Shin, J. S., Delamarre, L., Allen, P. & Mellman, I. (2008). Spinophilin participates in information transfer at immunological synapses. *J Cell Biol* **181**, 203-11.
89. Eun, S. Y., O'Connor, B. P., Wong, A. W., van Deventer, H. W., Taxman, D. J., Reed, W., Li, P., Blum, J. S., McKinnon, K. P. & Ting, J. P. (2006). Cutting edge: rho activation and actin polarization are dependent on plexin-A1 in dendritic cells. *J Immunol* **177**, 4271-5.
90. Riol-Blanco, L., Delgado-Martin, C., Sanchez-Sanchez, N., Alonso, C. L., Gutierrez-Lopez, M. D., Del Hoyo, G. M., Navarro, J., Sanchez-Madrid, F., Cabanas, C., Sanchez-Mateos, P. & Rodriguez-Fernandez, J. L. (2009). Immunological synapse formation inhibits, via NF-kappaB and FOXO1, the apoptosis of dendritic cells. *Nat Immunol* **10**, 753-60.
91. Hiraoka, S., Furumoto, Y., Koseki, H., Takagaki, Y., Taniguchi, M., Okumura, K. & Ra, C. (1999). Fc receptor beta subunit is required for full activation of mast cells through Fc receptor engagement. *Int Immunol* **11**, 199-207.

92. Bossi, G. & Griffiths, G. M. (1999). Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat Med* **5**, 90-6.
93. Jenkins, M. R., Tsun, A., Stinchcombe, J. C. & Griffiths, G. M. (2009). The strength of T cell receptor signal controls the polarization of cytotoxic machinery to the immunological synapse. *Immunity* **31**, 621-31.
94. Reichert, P., Reinhardt, R. L., Ingulli, E. & Jenkins, M. K. (2001). Cutting edge: in vivo identification of TCR redistribution and polarized IL-2 production by naive CD4 T cells. *J Immunol* **166**, 4278-81.
95. Huse, M., Lillemeier, B. F., Kuhns, M. S., Chen, D. S. & Davis, M. M. (2006). T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* **7**, 247-55.
96. Kay, J. G., Murray, R. Z., Pagan, J. K. & Stow, J. L. (2006). Cytokine secretion via cholesterol-rich lipid raft-associated SNAREs at the phagocytic cup. *J Biol Chem* **281**, 11949-54.
97. Manderson, A. P., Kay, J. G., Hammond, L. A., Brown, D. L. & Stow, J. L. (2007). Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. *J Cell Biol* **178**, 57-69.
98. Kupfer, A., Mosmann, T. R. & Kupfer, H. (1991). Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells. *Proc Natl Acad Sci U S A* **88**, 775-9.
99. Semino, C., Angelini, G., Poggi, A. & Rubartelli, A. (2005). NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood* **106**, 609-16.
100. Borg, C., Jalil, A., Laderach, D., Maruyama, K., Wakasugi, H., Charrier, S., Ryffel, B., Cambi, A., Figdor, C., Vainchenker, W., Galy, A., Caignard, A. & Zitvogel, L. (2004). NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. *Blood* **104**, 3267-75.
101. Gardella, S., Andrei, C., Lotti, L. V., Poggi, A., Torrisi, M. R., Zocchi, M. R. & Rubartelli, A. (2001). CD8(+) T lymphocytes induce polarized exocytosis of secretory lysosomes by dendritic cells with release of interleukin-1beta and cathepsin D. *Blood* **98**, 2152-9.
102. Gomez, T. S., Kumar, K., Medeiros, R. B., Shimizu, Y., Leibson, P. J. & Billadeau, D. D. (2007). Formins regulate the actin-related protein 2/3 complex-independent polarization of the centrosome to the immunological synapse. *Immunity* **26**, 177-90.
103. Stowers, L., Yelon, D., Berg, L. J. & Chant, J. (1995). Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42. *Proc Natl Acad Sci U S A* **92**, 5027-31.
104. Sancho, D., Nieto, M., Llano, M., Rodriguez-Fernandez, J. L., Tejedor, R., Avraham, S., Cabanas, C., Lopez-Botet, M. & Sanchez-Madrid, F. (2000). The tyrosine kinase PYK-2/RAFTK regulates natural killer (NK) cell cytotoxic response, and is translocated and activated upon specific target cell recognition and killing. *J Cell Biol* **149**, 1249-62.
105. Robertson, L. K., Mireau, L. R. & Ostergaard, H. L. (2005). A role for phosphatidylinositol 3-kinase in TCR-stimulated ERK activation leading to paxillin phosphorylation and CTL degranulation. *J Immunol* **175**, 8138-45.

106. Chen, X., Allan, D. S., Krzewski, K., Ge, B., Kopcow, H. & Strominger, J. L. (2006). CD28-stimulated ERK2 phosphorylation is required for polarization of the microtubule organizing center and granules in YTS NK cells. *Proc Natl Acad Sci U S A* **103**, 10346-51.
107. Stinchcombe, J. C., Barral, D. C., Mules, E. H., Booth, S., Hume, A. N., Machesky, L. M., Seabra, M. C. & Griffiths, G. M. (2001). Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J Cell Biol* **152**, 825-34.
108. Clark, R. H., Stinchcombe, J. C., Day, A., Blott, E., Booth, S., Bossi, G., Hamblin, T., Davies, E. G. & Griffiths, G. M. (2003). Adaptor protein 3-dependent microtubule-mediated movement of lytic granules to the immunological synapse. *Nat Immunol* **4**, 1111-20.
109. Neeft, M., Wieffer, M., de Jong, A. S., Negroiu, G., Metz, C. H., van Loon, A., Griffith, J., Krijgsveld, J., Wulffraat, N., Koch, H., Heck, A. J., Brose, N., Kleijmeer, M. & van der Sluijs, P. (2005). Munc13-4 is an effector of rab27a and controls secretion of lysosomes in hematopoietic cells. *Mol Biol Cell* **16**, 731-41.
110. Bryceson, Y. T., Rudd, E., Zheng, C., Edner, J., Ma, D., Wood, S. M., Bechensteen, A. G., Boelens, J. J., Celkan, T., Farah, R. A., Hultenby, K., Winiarski, J., Roche, P. A., Nordenskjold, M., Henter, J. I., Long, E. O. & Ljunggren, H. G. (2007). Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. *Blood* **110**, 1906-15.
111. Holt, O., Kanno, E., Bossi, G., Booth, S., Daniele, T., Santoro, A., Arico, M., Saegusa, C., Fukuda, M. & Griffiths, G. M. (2008). Slp1 and Slp2-a localize to the plasma membrane of CTL and contribute to secretion from the immunological synapse. *Traffic* **9**, 446-57.
112. Ma, J. S., Monu, N., Shen, D. T., Mecklenbrauker, I., Radoja, N., Haydar, T. F., Leitges, M., Frey, A. B., Vukmanovic, S. & Radoja, S. (2007). Protein kinase Cdelta regulates antigen receptor-induced lytic granule polarization in mouse CD8+ CTL. *J Immunol* **178**, 7814-21.
113. Das, V., Nal, B., Dujeancourt, A., Thoulouze, M. I., Galli, T., Roux, P., Dautry-Varsat, A. & Alcover, A. (2004). Activation-induced polarized recycling targets T cell antigen receptors to the immunological synapse; involvement of SNARE complexes. *Immunity* **20**, 577-88.
114. Dalod, M., Salazar-Mather, T. P., Malmgaard, L., Lewis, C., Asselin-Paturel, C., Briere, F., Trinchieri, G. & Biron, C. A. (2002). Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *J Exp Med* **195**, 517-28.
115. Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A. & Murphy, K. M. (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260**, 547-9.
116. Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-46.
117. Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R. & Trinchieri, G. (1996). The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J Exp Med* **183**, 147-57.
118. Aste-Amezaga, M., Ma, X., Sartori, A. & Trinchieri, G. (1998). Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol* **160**, 5936-44.

119. Trinchieri, G. (1998). Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* **70**, 83-243.
120. Yang, J., Murphy, T. L., Ouyang, W. & Murphy, K. M. (1999). Induction of interferon-gamma production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. *Eur J Immunol* **29**, 548-55.
121. Murphy, K. M. & Reiner, S. L. (2002). The lineage decisions of helper T cells. *Nat Rev Immunol* **2**, 933-44.
122. Chan, S. H., Kobayashi, M., Santoli, D., Perussia, B. & Trinchieri, G. (1992). Mechanisms of IFN-gamma induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J Immunol* **148**, 92-8.
123. Presky, D. H., Yang, H., Minetti, L. J., Chua, A. O., Nabavi, N., Wu, C. Y., Gately, M. K. & Gubler, U. (1996). A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci U S A* **93**, 14002-7.
124. Cho, S. S., Bacon, C. M., Sudarshan, C., Rees, R. C., Finbloom, D., Pine, R. & O'Shea, J. J. (1996). Activation of STAT4 by IL-12 and IFN-alpha: evidence for the involvement of ligand-induced tyrosine and serine phosphorylation. *J Immunol* **157**, 4781-9.
125. Prislovsky, A., Marathe, B., Hosni, A., Bolen, A. L., Nimmerjahn, F., Jackson, C. W., Weiman, D. & Strom, T. S. (2008). Rapid platelet turnover in WASP(-) mice correlates with increased ex vivo phagocytosis of opsonized WASP(-) platelets. *Exp Hematol* **36**, 609-23.
126. Marangoni, F., Trifari, S., Scaramuzza, S., Panaroni, C., Martino, S., Notarangelo, L. D., Baz, Z., Metin, A., Cattaneo, F., Villa, A., Aiuti, A., Battaglia, M., Roncarolo, M. G. & Dupre, L. (2007). WASP regulates suppressor activity of human and murine CD4(+)CD25(+)FOXP3(+) natural regulatory T cells. *J Exp Med* **204**, 369-80.
127. Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G. M., Nespoli, A., Viale, G., Allavena, P. & Rescigno, M. (2005). Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol* **6**, 507-14.
128. Allavena, P., Badolato, R., Facchetti, F., Vermi, W., Paganin, C., Luini, W., Giliani, S., Mazza, C., Bolzern, U., Chiesa, I., Notarangelo, L., Mantovani, A. & Sozzani, S. (2001). Monocytes from Wiskott-Aldrich patients differentiate in functional mature dendritic cells with a defect in CD83 expression. *Eur J Immunol* **31**, 3413-21.
129. Delon, J. (2000). The immunological synapse. *Curr Biol* **10**, R214.
130. Al-Alwan, M. M., Rowden, G., Lee, T. D. & West, K. A. (2001). The dendritic cell cytoskeleton is critical for the formation of the immunological synapse. *J Immunol* **166**, 1452-6.
131. Maldonado, R. A., Soriano, M. A., Perdomo, L. C., Sigrist, K., Irvine, D. J., Decker, T. & Glimcher, L. H. (2009). Control of T helper cell differentiation through cytokine receptor inclusion in the immunological synapse. *J Exp Med* **206**, 877-92.
132. Li, Q., Eppolito, C., Odunsi, K. & Shrikant, P. A. (2006). IL-12-programmed long-term CD8+ T cell responses require STAT4. *J Immunol* **177**, 7618-25.
133. Tsujita, K., Suetsugu, S., Sasaki, N., Furutani, M., Oikawa, T. & Takenawa, T. (2006). Coordination between the actin cytoskeleton and membrane

deformation by a novel membrane tubulation domain of PCH proteins is involved in endocytosis. *J Cell Biol* **172**, 269-79.

134. Jantsch, J., Cheminay, C., Chakravortty, D., Lindig, T., Hein, J. & Hensel, M. (2003). Intracellular activities of Salmonella enterica in murine dendritic cells. *Cell Microbiol* **5**, 933-45.
135. Smith, A. C., Heo, W. D., Braun, V., Jiang, X., Macrae, C., Casanova, J. E., Scidmore, M. A., Grinstein, S., Meyer, T. & Brumell, J. H. (2007). A network of Rab GTPases controls phagosome maturation and is modulated by Salmonella enterica serovar Typhimurium. *J Cell Biol* **176**, 263-8.
136. Kong, L. & Ge, B. X. (2008). MyD88-independent activation of a novel actin-Cdc42/Rac pathway is required for Toll-like receptor-stimulated phagocytosis. *Cell Res* **18**, 745-55.
137. Arbibe, L., Kim, D. W., Batsche, E., Pedron, T., Mateescu, B., Muchardt, C., Parsot, C. & Sansonetti, P. J. (2007). An injected bacterial effector targets chromatin access for transcription factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat Immunol* **8**, 47-56.
138. Celli, S., Lemaitre, F. & Bousso, P. (2007). Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4+ T cell activation. *Immunity* **27**, 625-34.
139. Scholer, A., Hugues, S., Boissonnas, A., Fetler, L. & Amigorena, S. (2008). Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory. *Immunity* **28**, 258-70.
140. West, M. A., Wallin, R. P., Matthews, S. P., Svensson, H. G., Zaru, R., Ljunggren, H. G., Prescott, A. R. & Watts, C. (2004). Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science* **305**, 1153-7.
141. Etienne-Manneville, S. & Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106**, 489-98.
142. Rodriguez-Fernandez, J. L., Gomez, M., Luque, A., Hogg, N., Sanchez-Madrid, F. & Cabanas, C. (1999). The interaction of activated integrin lymphocyte function-associated antigen 1 with ligand intercellular adhesion molecule 1 induces activation and redistribution of focal adhesion kinase and proline-rich tyrosine kinase 2 in T lymphocytes. *Mol Biol Cell* **10**, 1891-907.
143. Stewart, D. M., Tian, L. & Nelson, D. L. (2001). Linking cellular activation to cytoskeletal reorganization: Wiskott-Aldrich syndrome as a model. *Curr Opin Allergy Clin Immunol* **1**, 525-33.
144. Linder, S., Hufner, K., Wintergerst, U. & Aepfelbacher, M. (2000). Microtubule-dependent formation of podosomal adhesion structures in primary human macrophages. *J Cell Sci* **113 Pt 23**, 4165-76.
145. Blanco, P., Palucka, A. K., Pascual, V. & Banchereau, J. (2008). Dendritic cells and cytokines in human inflammatory and autoimmune diseases. *Cytokine Growth Factor Rev* **19**, 41-52.
146. Burkhardt, J. K. (1998). The role of microtubule-based motor proteins in maintaining the structure and function of the Golgi complex. *Biochim Biophys Acta* **1404**, 113-26.

147. Preisinger, C., Short, B., De Corte, V., Bruyneel, E., Haas, A., Kopajtich, R., Gettemans, J. & Barr, F. A. (2004). YSK1 is activated by the Golgi matrix protein GM130 and plays a role in cell migration through its substrate 14-3-3zeta. *J Cell Biol* **164**, 1009-20.
148. Chabin-Brion, K., Marceiller, J., Perez, F., Settegrana, C., Drechou, A., Durand, G. & Pous, C. (2001). The Golgi complex is a microtubule-organizing organelle. *Mol Biol Cell* **12**, 2047-60.
149. Efimov, A., Kharitonov, A., Efimova, N., Loncarek, J., Miller, P. M., Andreyeva, N., Gleeson, P., Galjart, N., Maia, A. R., McLeod, I. X., Yates, J. R., 3rd, Maiato, H., Khodjakov, A., Akhmanova, A. & Kaverina, I. (2007). Asymmetric CLASP-dependent nucleation of noncentrosomal microtubules at the trans-Golgi network. *Dev Cell* **12**, 917-30.
150. Hammond, J. W., Cai, D. & Verhey, K. J. (2008). Tubulin modifications and their cellular functions. *Curr Opin Cell Biol* **20**, 71-6.
151. Krzewski, K., Chen, X. & Strominger, J. L. (2008). WIP is essential for lytic granule polarization and NK cell cytotoxicity. *Proc Natl Acad Sci U S A* **105**, 2568-73.
152. Banerjee, P. P., Pandey, R., Zheng, R., Suhoski, M. M., Monaco-Shawver, L. & Orange, J. S. (2007). Cdc42-interacting protein-4 functionally links actin and microtubule networks at the cytolytic NK cell immunological synapse. *J Exp Med* **204**, 2305-20.
153. Morales-Tirado, V., Johannson, S., Hanson, E., Howell, A., Zhang, J., Siminovitch, K. A. & Fowell, D. J. (2004). Cutting edge: selective requirement for the Wiskott-Aldrich syndrome protein in cytokine, but not chemokine, secretion by CD4+ T cells. *J Immunol* **173**, 726-30.
154. Stow, J. L., Manderson, A. P. & Murray, R. Z. (2006). SNAREing immunity: the role of SNAREs in the immune system. *Nat Rev Immunol* **6**, 919-29.
155. Steinman, R. M., Hawiger, D., Liu, K., Bonifaz, L., Bonnyay, D., Mahnke, K., Iyoda, T., Ravetch, J., Dhodapkar, M., Inaba, K. & Nussenzweig, M. (2003). Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann N Y Acad Sci* **987**, 15-25.
156. Mahnke, K., Schmitt, E., Bonifaz, L., Enk, A. H. & Jonuleit, H. (2002). Immature, but not inactive: the tolerogenic function of immature dendritic cells. *Immunol Cell Biol* **80**, 477-83.
157. Moser, M. (2003). Dendritic cells in immunity and tolerance-do they display opposite functions? *Immunity* **19**, 5-8.
158. Hernandez, J., Aung, S., Marquardt, K. & Sherman, L. A. (2002). Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens. *J Exp Med* **196**, 323-33.
159. Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P. & O'Keeffe, M. (2001). Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *J Immunol* **166**, 5448-55.
160. Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K. & Mescher, M. F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol* **162**, 3256-62.
161. Goriely, S., Neurath, M. F. & Goldman, M. (2008). How microorganisms tip the balance between interleukin-12 family members. *Nat Rev Immunol* **8**, 81-6.

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