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Role of Polarity and Polarized Secretion in Dendritic Cells Functions

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Candidate: Jelena Petrovic

Supervisor: Dr. Federica Benvenuti

Contents

Introduction

1.1. Dendritic cells ***1***

1.1. 1. Dendritic cells general features *1*

1.1.2. Dendritic cells heterogeneity *3*

1.1.3. Immature DCs *6*

1.1.5. Role of DCs in priming adaptive immunity *10*

1.2. Cytoskeleton and polarity proteins ***12***

1.2.1. Cytoskeleton general features and role *13*

1.2.2. Polarity proteins general features and role *15*

1.2.2.1. Three polarity protein complexes - Par, Crumbs and Scrib *15*

1.3. Regulation of polarization ***21***

1.4. Cytoskeleton and polarity proteins in immune system ***23***

1.4.1. Cell migration *24*

1.4.1.1. The role of cytoskeleton in T cells migration *24*

1.4.1.2. The role of polarity proteins in T cells migration *25*

1.4.1.3. DCs migration *27*

1.4.2. Immunological synapse (IS) formation *28*

1.4.2.1. The role of cytoskeleton in T cells during IS *29*

1.4.2.2. The role of polarity proteins in T cell during IS *31*

1.4.2.3. DCs in synapse *33*

1.4.2.4. Polarized secretion *35*

Materials and Methods ***39***

Results ***47***

3.1. The functional aspect of MTOC polarization in DCs during synapse formation ***49***

3.1.1. DCs polarize MTOC/IL-12 toward antigen-specific synapse with T cells *49*

3.1.2. IL-12 signaling at the synapse *53*

3.1.3. WASp as a putative model to follow MTOC polarization *55*

3.1.4. Cdc42 regulates MTOC polarization toward the synapse *58*

3.1.5. Assessing the effect of Cdc42-mediate MTOC polarization in DCs on the functionality of T cells 61
3.2. Further into polarity-Disc Large protein (Dlg1) 65
3.2.1. Dlg1 localizes mainly in the membrane area in immature DCs..........65
3.2.2. Dlg1 undergoes relocalization from membrane toward the protrusions in mature DCs..........67
3.2.3. Dlg1 is phosphorilated upon TLR stimulation 68
3.2.4. Dlg1 polarizes toward the synapse in TLR and antigen dependent manner 70
3.3. Function of Dlg1 in DCs 72
3.3.1. Dlg1 and migration 73
3.3.2. Dlg1 in synapse formation and T cell functioning 75
3.3.2.1. Assessing the functional consequences of Dlg1 depletion in DCs on T cells functions 76
3.3.2.1.1. CD69 expression and IL-2 production in T cells stimulated by Dlg1-depleted DCs 76
3.3.2.1.2. INF-γ production in T cells stimulated by Dlg1-depleted DCs 78
Discussion 83
Conclusions 99
Bibliography 101

Abstract

A timely and spatially well-organized utilization of polarity determinants appears to be an absolute functional prerequisite of all cells, including leukocytes. Numerous *in vitro* and *in vivo* studies performed in the field of DCs biology have strongly stressed the idea that DCs ascertain a correct immune response by finely tuning signal transmission within IS and by temporally regulating contact duration with T cells. In this study we elucidate another important aspect of DCs biology as we demonstrate that TLR-stimulated DCs preferentially secrete cytokines (IL-12) within the IS. We provide further insights into the mechanism of IL-12 delivery at the synapse area as we show that it strictly relies on microtubule organizing center (MTOC) reorientation toward antigen-specific synapse. On the molecular level, we find that the phenomenon of MTOC polarization in DCs is regulated by Cdc42 (a Rho GTPase) and Wiskott-Aldrich protein (WASp). Interfering with MTOC polarization had drastic impact on IL-12-dependent events occurring very early following synapse formation: pSTAT4 phosphorilation and IFN-γ production. At later time points, the absence of MTOC reorientation reflected in the decreased survival of T cells and strong reduction in their IFN-γ-producing capacity. Polarity proteins belonging to three main families (Par, Crb and Scrib), have been shown essential in ensuring polarized state of various cells. We find that in immature DCs, disc large protein (Dlg) is preferentially clustered underneath the membrane area whereas it undergoes relocation and hyperphosporilation following TLR stimulation. During DCs-T cell contact, we observed that Dlg clusters at DC-T cell interface in antigen-specific manner. Dlg depletion in DCs markedly reduced early, synapse-specific IFN-γ mRNA production by T cells; that reflected in a decreased IFN-γ production by differentiated T cells at alter time-points. Collectively, our results revealed that polarized cytokine secretion and Dlg clustering at IS are features acquired by DCs through their maturation program and provide a proof of principle that T cells fate is highly controlled by DCs.

Introduction

1.1. Dendritic cells

The evolution of the immune system has been advancing toward the expansion in the number of available components and optimization of effector mechanisms, all with a goal of an improved protection against numerous pathogens in the environment. From the "traces" of an immune system in the form of phagocytes in *Protozoa*, *via* heightened specialization of the components of this system in *Pisces*, *Amphibia* and *Reptilia*, the evolution reached complex and pathogen specific immune system of *Mammalia*. Here, on the basis of functional criteria, this complex immune system can be divided into two distinct parts: innate and adaptive immune system. The components of the innate immune system (*i.e.* macrophages, neutrophilles, and system of complement) represent the first line of defense as they have a capacity to recognize pathogens and inhibit its further spreading by activating inflammatory reactions. However, the activation of a specialized and more efficient adaptive immune response is essential for complete protection from pathogens. Dendritic cells (*DCs*) represent a link between innate and adaptive immune response owing to their ability to recognize pathogens within the innate immune response and inform the components of adaptive immunity about the presence of infection by presenting pathogen-derived antigens (Ag) to T lymphocytes; a process necessary for adaptive immune response activation. Thus activated, the constituents of adaptive immune response clear the infection by either direct killing of infected cells (cytotoxic T lymphocytes, *CTL*), by opsonizing pathogens with antibodies (*B lymphocytes*) thus making them more susceptible to the action of phagocytes and by secreting soluble mediators (*i.e.* cytokines) that are crucial for spatio-temporal orchestration of immune response (helper T cells, *Th*) (Steinman and Hemmi, 2006).

1.1. 1. Dendritic cells general features

For proper immune response awakening, the internalization of antigens (*Ag*) and their presentation to T lymphocytes within immunological synapse (*IS*) is necessary. The cells bestowed with this function are called antigen presenting cells (*APC*). By many criteria, DCs represent the most efficient APC of the immune system (Itano and Jenkins, 2003).

DCs are a heterogeneous group of leukocytes originating from bone-marrow precursors. Within the first phase of their life, DCs (so called *immature DCs)* are strategically situated within

epithelial tissues at the organisms' frontiers, where they constantly patrol the environment for the presence of pathogens, owing to the expression of many receptors (*PRRs*, pattern recognition receptors) recognizing the evolutionary conserved patterns within pathogens (*PAMPs*, pathogen-associated molecular patterns). This strategical function, coupled with their high intrinsic capacity to internalize recognized microbes *via* endocytic/phagocytic pathway, makes them extremely predisposed for fast antigen recognition.

Once the invader is internalized, DCs enter into integrated developmental program called maturation. Within this program, DCs downregulate their endocytotic capacity and upregulate the expression of cytokines, the expression of molecules necessary for antigen presentation (MHC), and co-stimulatory molecules (CD80, CD86 and CD40) necessary to optimally activate T cells. Furthermore, these cells undergo a global redistribution of their actin cytoskeleton resulting in the acquirement of a migration capacity. Further changes in the expression of chemokine receptors and adhesion molecules induce migration of these pathogen-carrying DCs toward proximal lymph nodes (*LN*) where they present antigens to naïve T cells.

Within IS, DCs (*mature DCs)* adopt specific morphology and engage the adhesion molecules on T cell side necessary for the synapse preservation. At the same time, they present degraded pathogen sequences to naïve CD4+ and CD8+ T cells, inducing their activation and differentiation toward a specific T helper cell (*Th1* or *Th2*) or cytotoxic T cells (*CTL*) lineages, respectively (Reis e Sousa, 2004; Steinman and Hemmi, 2006) (Figure 1).

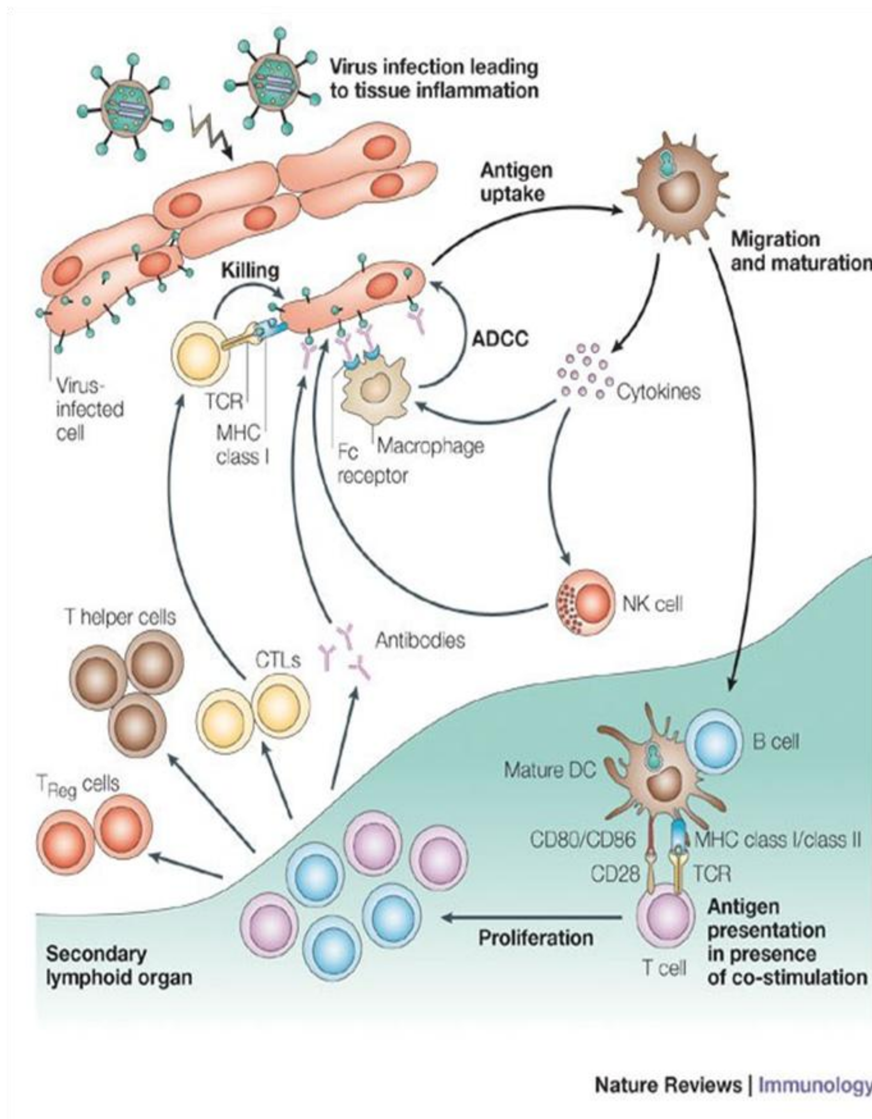

Figure 1. Dendritic cells (DCs) lifecycle. DCs are generally situated within the peripheral tissues where they have a pivotal role in recognizing the invading antigens. Once the antigen is recognized and internalized, DCs produce a high level of inflammatory cytokines essential to induce recruitment of cells specialized to induce inflammatory reactions (macrophages and neutrophilles) or direct killing of infected cells (NK cells). Concomitantly, DCs migrate toward a secondary lymph node where they present antigen-derived sequences to naïve T cells. This process initiates a cascade of events leading to activation and differentiation of T cells. Thus activated, T cells clear the infection by direct killing of infected cells (CTLs) and by secreting soluble mediators (cytokines) necessary to recruit other members of adaptive immune system (B cells) and to potentiate anti-pathogen activities of other cells (macrophages, DCs) (Th cells).

1.1.2. Dendritic cells heterogeneity

Currently accepted paradigm regarding DCs life cycle, suggests four stages of maturation *i.e.* functional characteristics of DCs: (i) *progenitors* from bone marrow (ii) *DCs precursors (preDCs),* which patrol through blood and lymphaticus and, upon contact with antigen differentiate into corresponding DCs (iii) *immature DCs*, localized within peripheral tissues, with great endocytotic and phagocytotic capacity that enables them to recognize and ingest antigens (*iv*) *mature DCs*, localized in the secondary lymphoid tissues, with a function in antigen presentation (Banchereau et al., 2000; Palucka and Banchereau, 1999)

The scheme given above is a representative of a general view of DCs life cycle, mostly known as *Langerhans cell paradigm*. In the light of this, however, one may think that the pathway from the pluripotent stem cell to the development of mature DC would be a straightforward process. However, a huge functional and phenotypical diversity of DCs found in lymph nodes and spleen challenges this simplistic theory of DCs ontogeny, reporting a vast degree of heterogeneity in all aspects of DCs life cycle: (i) *The precursors population*. DCs originate from both myeloid and lymphoid lineages. (ii) *Function*. DCs are responsible both for the activation of the immune response as well as for the induction of tolerance. (iv) *Anatomical localization*. DCs can be found in the peripheral tissues (Langerhans DCs, interstitial DCs), in spleen, in lymph nodes (interdigitate DCs), in liver and in blood (blood-derived DCs) (Banchereau et al., 2000; Naik et al., 2007; Shortman and Naik, 2007; Villadangos and Heath, 2005). There have been several trials to classify DCs according to various criteria that are still debated. In very general terms, DCs can be divided into two major groups: (i) non-lymphoid migratory DCs and lymphoid tissue resident DCs (ii) plasmacytoid DCs (*pDCs*) (Merad and Manz, 2009).

Non-lymphoid migratory DCs are mainly strategically localized in those tissues where they can quickly recognize the invading pathogens. Thus, they are found within tissues on the interface with the environment (lung, gut and skin) and at big filtering sites (liver and kidney). The prototype of this big family of DCs are Langerhans DCs (*LC*). They are easily distinguished by the surface expression of C-type lectin langerin and by LC-specific intracellular organelles known as Birbeck granules. Phenotypically, they are recognizable by a constitutive expression of major histocompatibility (MHC) class II complex and by low levels of CD11b. Furthermore, these cells express CD103 molecule, a ligand for the adhesion molecule E-cadherine expressed on most epithelial cells, which enables their retention within the tissues. LCs are discovered by Steinman in 1978 and Langerhans cell paradigm was postulated according to their functional characteristic (Gallegos and Bevan, 2006; Ginhoux and Merad, 2010).

Lymphoid tissue resident DCs are the most studied population of DCs. They are a very heterogeneous population that can be divided, based on the combination of phenotypical markers they express, in three major categories: $CD4^{+}CD8^{-}CD11b^{+}$, $CD4^{-}CD8^{+}CD11b^{-}$, and double negative $CD4^{-}CD8^{-}CD11b^{+}$cells. CD4+ DCs are mainly localized at the marginal zone and are specialized in MHC II presentation. CD8+ DCs are situated in T cell rich zones and were shown to be able to cross-present cell-associated antigens. The lymphoid resident DCs do not conform to the Langerhans cell paradigm as

they develop within the lymphoid organs directly from the precursors, without prior passing through peripheral tissues. In the absence of infection, these cells retain immature phenotype and are most likely involved in the maintenance of central tolerance. However, upon contact with pathogens, lymphoid DCs do acquire mature phenotype, secrete big amounts of inflammatory cytokines and migrate toward T cell rich zones. They have a role in activating adaptive immune response against blood borne antigens arriving at LN and spleen (den Haan et al., 2000; Wilson et al., 2003)

Plasmacytoid DCs were identified for the first time in human blood and lymphoid tissues. Due to their morphological similarity with plasma cells and expression of some T cell markers, they were initially named *plasmacytoid T cells* or *plasmacytoid monocytes* (Wu and Dakic, 2004). Later it was discovered that, following pathogen recognition, these cells obtain the ability to release high amounts of IFN-α and IFN-β; for this characteristic they were also named IFN-producing cells (*IPC*) (Colonna et al., 2004; Siegal et al., 1999). pDCs are specialized to combat against viral infections due to the expression of specific set of PRRs (TLR7 and TLR9), that recognize patterns within viral nucleic acids and stimulate the cascade of intracellular events resulting in IRF-7 induced production of IFN type I cytokines. In addition, viral recognition induces maturation of pDCs, visible in the upregulated expression of MHC class II molecules, co-stimulatory molecules (CD80, CD86 and cD40) and release of proinflamatory cytokines (IL-6 and TNF-α) (Dai et al., 2004).

Finally, DCs can be differentiated *in vitro* (*in vitro generated DCs*) owing to the existence of well-established methods. The first method reports that DCs can be generated from bone-marrow or spleen precursors in medium supplemented with granulocyte-monocyte colony stimulating factor (*GM-CSF*) with or without interleukin-4 (*IL-4*). Thus obtained cells are partially similar to some lymphoid organ resident DCs, as determined by phenotypical markers examination (Shortman and Naik, 2007). A more precise way to differentiate lymphoid organ-like DCs from bone-marrow precursors is by supplementing the medium with FMS-like tyrosine kinase 3 ligand (*Flt3L*). These DCs expressed phenotypical and functional characteristics equivalent to CD8+ and CD8- DCs: CD11b, CD24 surface marker expression; expression of mRNA for TLRs and chemokines receptors; the ability to produce cytokine (IL-12 and IFN) following TLR engagement and the ability to cross-present antigens (Naik et al., 2005).

1.1.3. Immature DCs

Immature DCs express sets of chemokine receptors (CCR1, CCR2, CCR4, CCR5, CXCR1 and CXCR4), which enable their specific localization within the peripheral epithelial tissues (Banchereau et al., 2000) The term *immature* is related to the incapacity of these cells to induce T cells proliferation, but they have a great capacity to recognize and engulf pathogens (Reis e Sousa, 2006). Immature DCs employ three major endocytotic mechanisms of pathogens internalization: phagocytosis, macropinocytosis and receptor-mediated endocytosis.

Antigen internalization

Phagocytosis. Phagocytosis represents most prevalent mechanism for pathogen engulfment. One of the reasons for that probably lies within the fact that this mechanism is used for multitude of processes. For instance, it includes receptor (FcγR, complement and lectin) mediated phagocytosis of extracellular pathogens; it is used as a main mechanism for sampling and presentation of the apoptotic bodies within the steady-state conditions (and thus tolerance maintenance); it is employed in sampling foreign antigens by engulfing the cells died from the infection; it enables inert particles and liposomes uptake (Trombetta and Mellman, 2005).

Once recognized, pathogens are uptaken through a formation of cytoskeleton-based membranous protrusions, wrapping in the pathogen within a vesicle. A special sophistication of this mechanism lies within a fact that different regulatory pathways and different types of membranous protrusions are activated depending on the nature of pathogen being internalized. Thus, phagocytosis through FcγR mediation induces Cdc42-dependent formation of pseudopodes while Rac activation is necessary for phagosome closure. On the other hand, complement mediated phagocytosis is Rho GTPase dependent. It does not include the formation of pseudopodes but rather involves integrin mediated ligation for the formation of phagosomes (Symons and Rusk, 2003).

Macropinocytosis. As its name suggests (from Greek *pinein-"*to drink"), this type of endocytic process is involved in sampling soluble antigens by uptaking huge amounts of fluidic fractions. The base of this process is Cdc42-regulated formation of huge membranous protrusions, encircling a big proportion of extracellular fluids and their fusion with the membrane. It is a constitutive process within immature DCs and is downregulated upon maturation (Conner and Schmid, 2003; Symons and Rusk, 2003).

Receptor mediated endocytosis. Mechanistically, receptor mediated endocytosis could be seen as the further refinement of general process of macropinocytosis. It is involved in sampling soluble antigens, but the presence of very sensitive pathogen-pattern specific receptors enables the cell to recognize pathogens that are present in extremely low concentrations. For that purpose DCs express the array of receptors able to perform endocytosis of antigens: (i) Receptors that recognize Fc region of immunoglobulins (*FcRs*) and complement receptors (*CRs*) are used to internalize immunoglobulin opsonized antigens (Fanger et al., 1996). (ii) C type lectin receptor that recognizes carbohydrate remnants on the surface of many pathogens (Steinman and Hemmi, 2006) (iii) a few of not well characterized receptors (*CD36*, *αβ3* or *αvβ5* integrins) for internalization of apoptotic and necrotic cells (iv) Scavenger receptors used to recognize low density lipoproteins (*LDLs*) (Delneste et al., 2002).

Antigen processing and presentation

Upon internalization, antigens are degraded into small immunogenic epitops that associate with MHC and are transported to the plasma membrane where they trigger the activation of naïve T cells. Generally accepted view is that exogenous antigens are presented within MHC class II (*MHC II*) complex while endogenous antigens are presented within MHC class I (*MHC I*), activating CD4+ and CD8+ T cells, respectively.

Specifically, MHC II are constantly being synthesized in immature DCs and accumulate in the form of trimer with the invariant chain (*Ii*) within specific, multilamelar structures, called *MHC II rich compartments*. These structures are involved in the endocytic pathway as they fuse with endocytic vesicles carrying exogenous antigen. The presence of Ag causes the exchange of the invariant chain with the antigen and its presentation on the DCs surface. Immature DCs express low levels of short-lived MHC II molecules on their surface. However, upon antigen recognition, MHC II-Ag complexes are stabilized and remain on DCs surface for couple of days, thus giving the time to rare Ag-specific CD4+ T lymphocytes to recognize them and initiate a series of proliferation and differentiation processes (Trombetta and Mellman, 2005).

On the other side, intracellular Ags are processed within MHC class I pathway. In short, endogenous antigens are ubiquitinated and, following the transport through proteaosome, degraded to a certain length (6-30 amino acid long), characterized by the presence of basic or hydrophobic amino acids on their carboxyl terminus. Oligonucleotides of this length and specificity are recognized by transporter associated with antigen presentation (*TAP*), which translocates them inside endoplasmic

reticulum (*ER*) where they associate with *de novo* synthesized MHC I dimer. Exocytotic vesicles carrying these trimers bud from the ER and fusing with plasma membrane, enable the presentation of antigens within MHC I complexes. Thus expressed antigens are being recognized by CD8+ T lymphocytes (Rock et al., 2004; Trombetta and Mellman, 2005).

However, this general view has been very shaken by the discovery that DCs can present exogenous antigens within MHC class I pathway (a process termed *cross-presentation*) very efficiently. Cross-presentation has been shown to enable the activation of CD8+T cells in response to either bacterial or viral stimulus. Furthermore, cross-presentation was shown to be important against pathogens which do not infect DCs (den Haan and Bevan, 2001) and in the presentation of self antigens; coming from the cells that died by apoptosis as a part of normal cellular turnover (necessary for peripheral tolerance maintenance) (Heath and Carbone, 2001) . However, mechanisms that DCs employ in order to perform this function is still elusive. It has been suggested to involve exogenous antigen "escape" from the phagosomes and its leakage into the cytosol; where it is recognized as foreign by the components of MHC class I-pathway (Monu and Trombetta, 2007).

1.1.4. Pathogen recognition and TLRs

At the same time that pathogen is being recognized and internalized by endocytotic machinery, another group of molecules expressed by immature DCs recognizes antigens. These molecules belong to pathogen recognition receptors family (*PRRs*), as they recognize the evolutionary conserved sequences invariably present on the surface of every pathogen (*PAMP*, *pathogen associated molecular pattern*) (Akira et al., 2001).

The best described group of PAMPs is the family of Toll like receptors (*TLR*). This family consists of about 12 known receptors that are different in their pattern of expression as well as in their specificity for the pathogens. Thus, some of them (TLR1, 2, 4, 5 and 6) are expressed on the cell surface where they mainly recognize bacterial products, while others (TLR3, 7, 8 and 9) are located on the membranes of the endocytic vesicles and are specialized in recognition of viral products. For instance, TLR4 is shown to recognize lipopolysaccharides (*LPS*), a major component of Gram^{-} bacteria wall; TLR2 recognizes bacterial peptidoglycans and lipoproteins; TLR5 recognizes bacterial flagellin; TLR7 and TLR9 recognize CpG-rich islands of bacterial and viral DNA (Matzinger, 2002).

Furthermore, the complexity is increased by the ability of some of these receptors to form heterodimers, thus increasing the sensitivity of the response (Takeda et al., 2003).

Following pathogen recognition, TLRs were shown to be able to activate two independent signaling pathways. The first pathway relies on the association with an adaptor molecule, MyD88, *via* its C-terminal domain. On the other side, Myd88 engages a kinase IRAK, whose consequent phosphorilation induces further association with TRAF6. The final outcome is the activation of two different signaling cascades: JNK and NF-κB. The second pathway evoked by TLR-mediated antigen recognition also results in the activation of NF-κB signaling pathway; however it involves a different adaptor molecule, namely TRIF (that further activates RIP1 and TRAF6). At the same time, however, TRIF was shown to induce the expression of IFN type I genes as it associates with two kinases, TBK1 and IKKi who further mediate phosphorilation-dependent activation of IFN-regulatory factor 3 (IRF3) ((Janeway and Medzhitov, 2002; Trinchieri and Sher, 2007).

From their side, these transcriptional factors ensure a complete turnover in DCs genetical program. Dendritic cells lose their capacity to internalize antigens due to a decrease in the expression of receptors performing antigen uptake and downregulation of endocytotic activity (Garrett et al., 2000). The expression of genes for cytokines, that indirectly induce inflammatory reactions by recruiting specific cells of the innate immunity (*i.e.* macrophages, neutrophilles,) is enhanced. These cytokines are also secreted later within immunological synapse where they are crucial for differentiation of T cells toward a specific linage. Furthermore, the expression of genes for co-stimulators (CD40, CD80 and CD86) is augmented, whose function in ensuring signal amplification is essential for the proper presentation of antigens in IS (Janeway and Medzhitov, 2002). Finally, inflammatory stimuli turn off DCs' response to those chemokines responsible for DCs retention within tissues, by negative autocrine effect or by receptor desensitization (Sallusto et al., 1998). At the same time, the expression of another group of chemokine receptors, namely CCR7, is upregulated, enabling these cells to respond to corresponding CCL19 and CCL21 chemokines. Taking into account that these chemokines are expressed in T cell zones of lymph nodes, the change in chemokine expression profile allows DCs to leave peripheral tissues and migrate in the direction of chemokine gradient- *i.e.* toward lymph nodes (Alvarez et al., 2008; Dieu et al., 1998).

1.1.5. Role of DCs in priming adaptive immunity

At present, the idea that the transmission of the signal between Ag-bearing DCs and T cells occurs during their contact in the lymph node *via* a specialized structure called immunological synapse (IS), is a well established paradigm. Later *in vivo* studies have further revealed the complexity of the interactions and demonstrated that a refined and dynamical interplay between DCs and T cells is a prelude for the formation of informational synapse (Bousso, 2008). Once the stable contact is formed, DCs deliver three types of signals that directly influence the outcome of the immune response. The first signal occurs upon interaction between TCR and MHC-bearing antigen which secures Ag-specific response. The second signal consists of the engagement of co-stimulatory molecules on DCs side by either CD28 or CTLA-4, which induces activation or inhibition of T cell activation, respectively. The third signal is mirrored in the combined effect of various cytokines secreted by DCs and integrated by T cell as the signals for proliferation and differentiation toward specific subsets (Kalinski et al., 1999b; Villadangos and Schnorrer, 2007).

The direct cause of T cell fate determination is given by the integral message of cytokines ("third signal") being secreted toward T in synapse with DCs. However, the decision regarding the nature of immune response being awakened has been decided much earlier, upon first contact of DCs with the pathogen. Specifically, pathogens engage a specific combination of TLRs present on the surface of immature DCs which further signal DCs to enter into a certain program of maturation, each of which results in the specific combination of cytokines being produced. Based on the type of antigen being recognized, DCs may prime the differentiation of T cells toward Th1, Th2, Th17 or Treg lineages (Reiner, 2007).

Th1 subset has specialized for the defense against intracellular bacteria and viruses. The activation of TLR3, TLR4, TLR7 or TLR9 on DCs surface induces the activation of DC maturation program characterized by the production of IL-12 cytokine. This cytokine induces IFN-γ production in T cells through the activation of special combination of signaling molecules (JAK2) and transcriptional factors (STAT4). Once produced, IFN-γ further polarizes the response toward Th1 dominated response by enhancing further IL-12 production in DCs and by sensitizing activated T cells to the effect of IL-12 (by upregulating the expression of IL-12β2R subunit on their surface) (Reiner, 2007; Szabo et al., 2000).

Th2 dominated response is specialized for the defense against helminthes and various allergens. TLRs being engaged by these microbes are still unknown, while the outcome of their

recognition by DCs is manifested in high production of IL-4. IL-4 induces the polarization of activated T cells toward Th2 dominated response by upregulating the expression of a transcriptional factor GATA3. Targets of this transcriptional factor are genes for IL-4 and IL-5 cytokines. These cytokines cause a complete polarization toward Th2 pathway by suppressing the expression of factors critical for the development of Th1 response.

Th17 are fairly recently discovered subtype of CD4+ T cells that have probably evolved to enhance the clearance of pathogens distinct from those targeted by Th1 and Th2. Their role has been implicated in defense against extracellular bacteria (*Klebsie pneumoniae*) and fungi (*Candida albicans*). The receptors being engaged by DCs in the presence of these pathogens is largely unknown, but initial reports by Reis e Sousa implicate the activation of dectin-1-Syk-CARd9 signaling pathway. DCs that recognize these pathogens secret a specific combination of cytokines having a role in inducing inflammatory reaction (IL-6 and TNFα) or polarizing Th response toward a Th17-dominated one (IL-23). The necessity of regulatory T cells (Tregs) and TGFβ in the polarization of the response toward Th17 dominated lineage has also been reported. This is in line with the reports suggesting that IL-6 and TGFβ are necessary to induce the activation of Th17 cells, while the presence of IL-23 secreted by DCs induces complete polarization of this response (Huang et al., 2004; Reiner, 2007; Weaver et al., 2006).

Regulatory T cells (*Treg*) develop from naïve CD4+T cells through their contact with DCs that have previously been exposed to cytokines IL-10 and TGFβ. The development of Tregs is associated with the maintenance of peripheral tolerance through a suppression of unwanted adaptive immune response and autoimmunity prevention. Regulatory T cells that develop at the periphery through DC-mediated contact (so called *adaptive T reg*) are different than naturally occurring Tregs developing in thymus. Within the population of adaptive Tregs, two main, functionally different populations can be distinguished. Treg1 develop upon the contact with IL-10-conditioned DCs and are characterized by the lack of transcription factor Foxp3 and production of large amounts of IL-10. Treg2 develop as the result of TGFβ signaling, they express Foxp3 and are functionally indistinguishably from intrathymical Tregs (Hall et al., 2011; Reiner, 2007) (Figure 2).

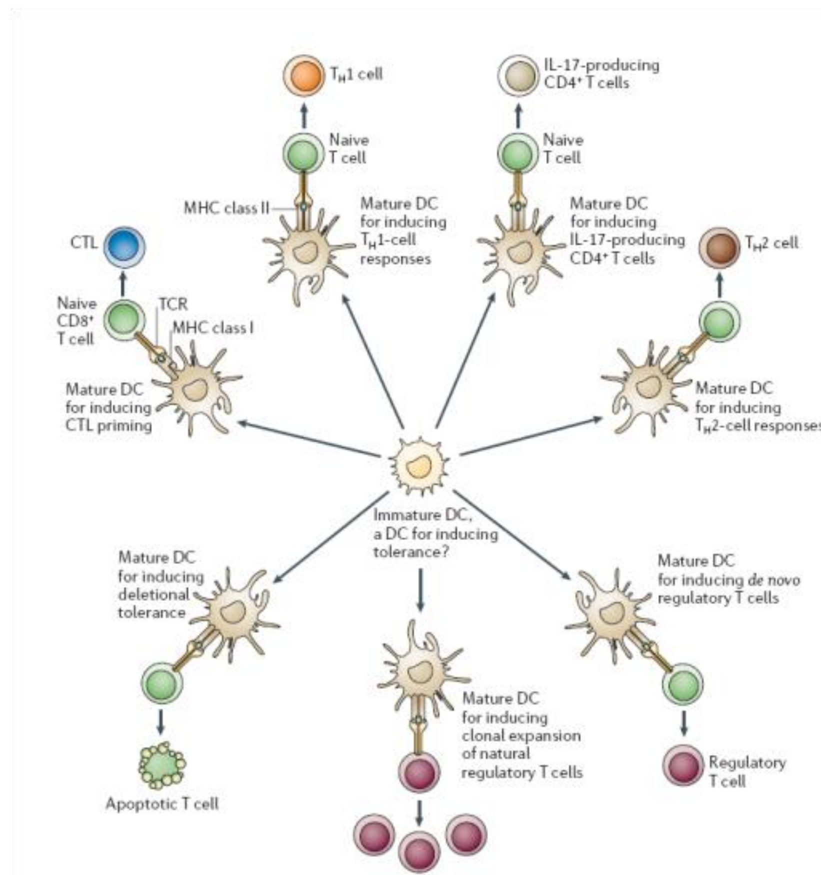

Figure 2. Dendritic cells effector functions. DCs maturation program is a process where DCs acquire phenotypical and functional characteristics necessary to present internalized antigens to naïve T cells. However, the outcome of DCs priming is very heterogeneous as DCs can potentiate the development of various lineages of T cells. This capacity of DCs depends on the type of pathogens being recognized by DCs, the environmental clues or DCs ontogeny (predisposing a certain pool of DCs toward a specific pathway). A direct cause of diverse T cell fate during priming by DCs relies on different sets of cytokines being secreted by DCs in response to various pathogenic circumstances promoting DCs maturation (Reis e Sousa, 2006).

1.2. Cytoskeleton and polarity proteins

At present, an emerging concept indicating that spatio-temporal interplay between numerous polarity players shapes cells' destinies and ensures their function becomes more evident. Current knowledge regarding polarity determined processes within cells has helped divide polarity determinants into arbitrary five categories actin cytoskeleton 2) microtubule cytoskeleton 3) surface proteins (receptors and lipid rafts) 4) secretion pathways (endo- and exocytosis) 5) polarity proteins complexes. In the following few chapters we will try to briefly communicate how dynamical changes of

cytoskeleton and mutual distribution of polarity proteins enable various aspects of cell functioning (Krummel and Macara, 2006).

1.2.1. Cytoskeleton general features and role

Whether stationary cells (e.g. epithelial cells), highly motile cells (e.g. neutrophilles, fibroblasts, astrocytes), cells with high proliferative capacity (e.g. embryonic stem cells) or the cells forming the synapse (e.g. lymphocyte, dendritic cells, neurons); all functions of these cells depend on the presence of stable and dynamic cytoskeleton architecture at the same time. This cytoskeleton networks needs to be firm in order to ensure consistency of the cellular processes in time; and it needs to be dynamic in order to meet cellular morphological prerogatives in response to various clues from the environment.

All cells studied possess three types of cytoskeleton elements: actin, microtubules and intermediate filaments. So far, the role of intermediate filaments has not been implicated in polarity processes regulation, while actin and microtubules have long been known for their role in supporting functional dynamism of cells. Moreover, until recently, it has been thought that actin and microtubules react independently to changes in intracellular and extracellular milieu; however, recent advances have made it clear that adequate cellular functioning is a consequence of integrated action of these two networks (Banerjee et al., 2007; Kodama et al., 2004; Rodriguez et al., 2003).

Actin is usually present within cells in the form of F-actin (*filamentous actin)*, a polymer composed of only one type of monomer, G-actin (*globular actin*). The process of actin nucleation depends on the presence of Arp2/3 complex that stimulates G-actin incorporation. Cycle of actin polimerization and depolimerization, regulated by ATP hydrolysis and assisted by accessory proteins enable the cells to respond to various signals by obtaining adequate morphology. Microtubule (MT) network is preferentially structured as a polymeric network of α and β-tubulin spanning radially from a core consisting of two centriols and pericentriolar material (*PCM*; consisting mainly of γ-tubulin monomers). This central structure was termed microtubule organizing center (*MTOC*) also known as *centrosome.* MT polimerization from this center is characterized by growth of one end (*plus end*) of MTs, while the other end remains attached to MTOC (*minus end*) (Gundersen, 2002; Rodriguez et al., 2003).

Actin and MTOC have been implicated, as mentioned, in various cellular functions. For instance, during cell migration, changes in the composition in extracellular environment induce actin

recruitment toward cell periphery and MTOC reorientation toward one pole of the cell. Actin subcortical clustering is crucial for cellular migration as it ensures a stability of cells' migration shape and it represents a base of membrane protrusions *e.g.* filopodia, lamellipodia (Hall, 1998); while MTOC translocation toward one pole is important to bring the directionality of the movement (Kodama et al., 2004). Moreover, adequate spindle orientation is important for the proper segregation of fate determinants and consequential asymmetrical division during *D.melanogaster* and mouse neurogenesis (Knoblich, 2008). In mouse embryonic hippocampus neurons, polarization of MT and actin cytoskeleton within one of the numerous membrane protrusions (*minor processusses*) predestines it to become axon (Arimura and Kaibuchi, 2007). Finally, apico-basal polarity of epithelial cells is a result of junction complexes being held firmly in their places by a meshwork of actin cytoskeleton (Knust and Bossinger, 2002) (Figure 3).

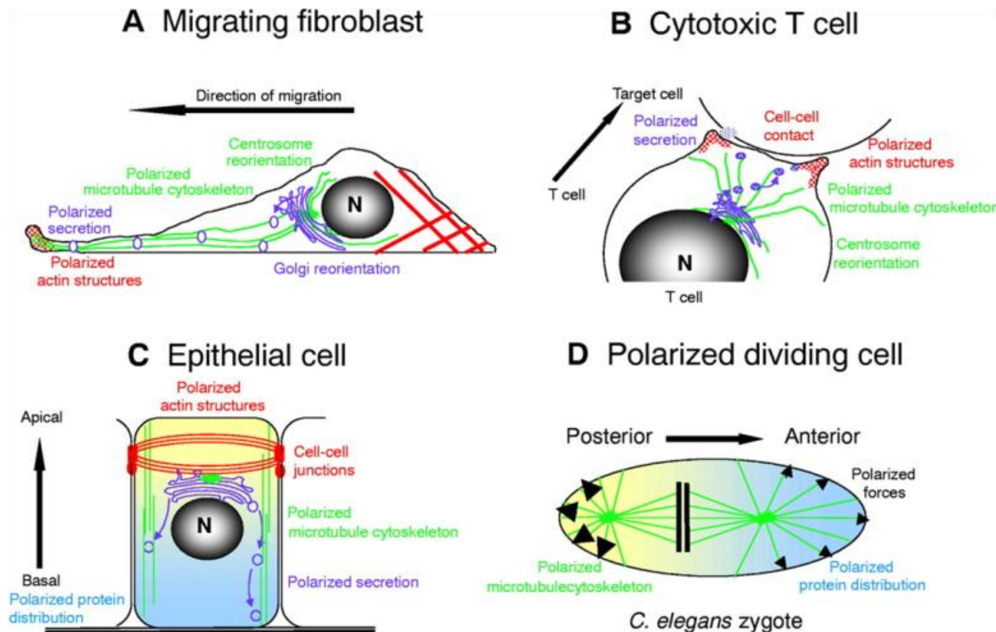

Figure 3. Role of cytoskeleton in various cells. a) In motile cells, actin cytoskeleton polarization toward the leading edge enables formation of membranous protrusions (lamelopodia and filopodia); while centrosome positioning in the rear end probably contributes to the directionality of the movement. b) Polarized secretion of soluble mediators in cytotoxic T cells is ensured by centrosome polarization toward the synapse region. Docking of plus-end MTs at the outskirts of synapse represents a pathway for vesicle transport. c) Functionally important apico-basal polarity of epithelial cells is a result of junction complexes held together by the inner actin cytoskeleton network. MTOC (centrosome) has a function in directing polarized secretion of vesicles toward the specific region of epithelial cells. d) In dividing *C.elegans* zygote, spindle positioning establishes the axis of polarity, ensures unequal distribution of other fate determinants and enables asymmetrical cell division (Etienne-Manneville, 2004).

1.2.2. Polarity proteins general features and role

First results considering phenomenon of polarity came about the end of the 19th century but they were restricted to the mere morphological descriptions of asymmetrically distributed components within cells and tissues. With the first postulated model on the topic of asymmetrical cell divisions in ascidian egg back in 1905 (Conklin, 1905), the idea that the relative distribution of proteins within a cell may play an important role in cell destiny came to light. It was not until the end of 20th century, however, that the first determinant of segregation was molecularly characterized in the sensory organ precursors (SOP) in peripheral nervous system of *D.melanogaster* (Rhyu et al., 1994). Asymmetrical inheritance of cell fate determinants was then also shown to induce emergence of diverse embryonic tissues in *C.elegans* (Betschinger and Knoblich, 2004). Studies in mammalian cell systems have demonstrated that asymmetry seems to be a common characteristic of all multicellular organisms, rather than the exception (Horvitz and Herskowitz, 1992). For instance, the presence of asymmetrical division is evident also in mouse zygote (Knoblich, 2008); formation of transient polarity modules represents the base of cellular locomotion (Etienne-Manneville, 2008); polarized clustering of specific proteins in the region of immunological synapse ensures proper signal transmission between the cells (Krummel and Macara, 2006).

Further reports aimed to understand the mechanisms and players involved in establishing cellular polarity were mainly performed on epithelial tissues. Intensive research in this field has revealed the existence of evolutionary conserved group of proteins with a role in ensuring apico-basal organization of cells. Following these observations the proteins were classified within specific polarity complexes and the term *polarity protein* was created.

1.2.2.1. Three polarity protein complexes - Par, Crumbs and Scrib

PAR complex

The name *PAR* stands for *partition defective*, the term that crystallized itself upon early works in *C.elegans*, where it was shown that mutants lacking *par* genes experienced defects in division patterns during *C.elegans* development (Kemphues et al., 1988). In the same work, genetic analysis revealed six *par* genes (*i.e.* proteins) responsible for this phenomenon: *par1, par2, Par3, par4, par5* and *par6.* Through sequence alignment it was observed that *C.elegans* Par6, *D.melanogaster* dmPar6 and mammalian Par6 have a huge extent of homology (Joberty et al., 2000). Molecular characterization has

demonstrated that *C.elegans* Par3 protein is a *D.melanogaster* protein Bazook (one of the main players that leads to the proper asymmetrical division in *D.melanogaster*) homologue (Kuchinke et al., 1998). The addition of another protein, aPKC, shown to be physically and functionally connected with Par proteins in *D.melanogaster* epithelia, formed the Par trilogy complex known as Par3/Par6/aPKC complex (Suzuki et al., 2001).

General mechanism of action: Although the meaning of polarity diverges with the respect to cell types and organisms involved, the general mode of Par protein functioning seems to be preserved. This protein complex, in invertebrates as well as in vertebrates, has been shown to contribute to the establishment of epithelial cell polarity, through its role in apical domain and *zonula adherens* (*ZO*) formation. In these cells, Par6 seems to have the initial role in scaffolding other proteins (Joberty et al., 2000; Suzuki and Ohno, 2006). Specifically, Par6 receives a message regarding the change in polarity cues through the action of a small RhoGTPase-Cdc42. This signal is then translated into inner polarization signal by Par6 binding to aPKC (Joberty et al., 2000; Lin et al., 2000). Interestingly, inactive aPKC is found associated with a protein from another polarity complex, Lgl (*lethal giant larvae)* (Plant et al., 2003). Par6 binding induces aPKC phosphorilation-dependent dissociations from this complex and its adherence to Par3. Initially, this association induce the formation of the final Par6/Par3/aPKC complex (Yamanaka et al., 2006; Yamanaka et al., 2003). However, at the same time, Par3 phosphorilation by aPKC induces the loss of Par3 affinity for the complex and its disassociation (Nagai-Tamai et al., 2002). This is in line with some later reports stating that Par3 and aPKC/Par6 complex are not always found co-localizing in epithelial cells (Harris and Peifer, 2005). Moreover, it appears that exactly this dynamical association between the members of this complex is fundamental for adequate polarity pattern development of epithelial cells. Specifically, Par6/Par3/aPKC association was shown to be important for the formation of apical domain in epithelial cells, whilst its dissociation is important for the tight junction formation (Horikoshi et al., 2009; Nagai-Tamai et al., 2002). Overall, this prototype of temporary connections between Par polarity complex proteins enabling cell morphology has found its proof of principle in various cell types (McCaffrey and Macara, 2011).

Crumbs (Crb) complex

This complex originally got its name from morphological appearance of *D.melanogaster* cuticle, resulting from the absence of the gene *crumbs* in the epithelial cells underneath that secrete the cuticle (Tepass et al., 1990). Further studies on the nature of this protein revealed that Crumbs is

responsible for apical domain maintenance of epithelial cells in *D.melanogaster*, as its overexpression in *crb* mutants restored the apical phenotype (Wodarz et al., 1995). Afterwards, functional colocalization of Stardust (Std) and Crumbs in the adequate organization of the apical domain of epithelial cells was demonstrated (Bachmann et al., 2001; Hong et al., 2001). Finally, a third protein whose depletion induced complete perturbation of the epithelial cell polarity, as well as Crb mislocalization, was revealed (Bhat et al., 1999). The name of this protein is Disc Lost (Dlt), and that marked the formation of another polarity complex. All these proteins, through various and multiple studies have found their homologues in vertebrates. Thus, the appropriate counterparts of these three proteins in mammals are: Crb1, Crb2 and Crb3 (homologues of Crb), PALS1 (homologue of Sdt) and PATJ/MUPP1 (homologues of Dlt) (Assemat et al., 2008).

Mechanisms of action: Most of the data regarding the function of this complex comes from works in mammalian epithelia. As deciphered from these studies Crb, Sdt and Dlt are all physically associated and are responsible for the formation of apical domain and tight junction maintenance in these cells. Specifically, Roh *et.al.,* were able to see that PALS1 was indirectly targeted toward the tight junction through its association with a 200kDa protein which, upon sequencing, turned out to be PATJ (Roh et al., 2002). In the same study, fluorescent tracking of Myc-Crb1 within MDCK cells revealed that this protein localizes at tight junctions where it makes a ternary complex with both PALS1 and PATJ. Moreover, depletion of PALS1 induces perturbation not only of PATJ and Crb3, but also of two other proteins specifically found within tight junctions: occludin and ZO-3 (Michel et al., 2005). Finally, the association between these three proteins was shown to have functional consequences on the apical domain identity as the apical zone overgrowth was noticed in the cells in which Crb was overexpressed (Roh et al., 2003). The role of the ultimate member of this complex, MUPP1, remains still elusive. Its relation to the Crb complex was demonstrated since its L27 domain was shown to bind to PALS1 (Roh et al., 2002). However, its role in the formation/maintenance of the epithelial cell polarity still remains to be explored.

Scrib complex

This third complex consists of three proteins: Scrib, Dlg (*Disc Large protein*) and Lgl (*Lethal Giant Larvae protein*). Two of these proteins, Dlg and Lgl, were initially isolated from the tumors in imaginal disk epithelia of *D.melanogaster*. Specifically, the mutations in these two genes caused the tumoral overgrowth of imaginal epithelia and consequential death of *D.melanogaster* larvaes

(Gateff, 1978); hence, they were categorized as tumor-suppressor proteins. Scrib was initially mapped in a screen for the genes whose mutations affect the adequate morphogenesis of epithelial cells in *D.melanogaster* embryos. Specifically, the cuticle (a product of secretion of epithelial cells beneath it) of *scrib* mutants was found wrinkled and intensively perforated. On the other side, the cuticle of wild-type embryos had an even and immaculate appearance (Bilder and Perrimon, 2000). Thus, these three proteins got their names based on these initial observations regarding the phenotype they cause when mutated. All of these proteins found their orthologues in mammals (Dow et al., 2003; Lue et al., 1994).

Scrib. Scrib is a cytoplasmic multidomain scaffolding protein belonging to the LAP protein family (Assemat et al., 2008; Santoni et al., 2002). Even though the role of Scrib in the formation of cell junction complexes has long been established for *D.melanogaster* epithelial cells, its role in the mammalian counterparts was proven much harder to elucidate. One of the first results regarding its role in the mammalian epithelia came from few contemporaneous studies. Specifically, Navarro C *et.al.* have shown that hScrib is enriched at the adherens junctions (AJ) in MDCK cells, as demonstrated by fluorescent staining and the loss-of-functions experiments (Navarro et al., 2005). Functional contribution of Scrib to overall epithelial cell polarity was shown by Zeitler J *et.al.* who demonstrated that cells transfected with *scrib* mutant for one of the domains (LRR) failed to localize Scrib toward junction complexes and caused actin cytoskeleton displacement, epithelial cell rounding and uncontrolled proliferation (Navarro et al., 2005; Zeitler et al., 2004). Immediately after, Qin Y *et.al.,* further broadened its function by demonstrated that hScrib does not only regulate the adhesion between MDCK cells, but their migration as well. Specifically, MDCK cell in which hScrib has been depleted were shown to express increased motility as well as loss of directionality (Qin et al., 2005). Additionally, hScrib was also shown to negatively regulate cell cycle progression by its localizing in the area of basolateral junction complexes (Nagasaka et al., 2006). For its role in negatively affecting migration and proliferation, Scrib was suggested to belong to a group of tumor suppressors. This was additionally confirmed by many studies that correlated the effect of Scrib absence to the emergence of tumorogenic phenotype (Gardiol et al., 2006; Nakagawa et al., 2004; Nguyen et al., 2003).

Dlg. Dlg is a protein belonging to a membrane associated guanilate kinase (MAGUK) family, a family that functions as a scaffold for other proteins. Dlg is represented by four homologues in mammals: Dlg1-Dlg4, out of which Dlg1 is the one with most widespread distribution within the tissues as well as closest homolog to *D.melanogaster* Dlg (Assemat et al., 2008)

The initial data suggesting that Dlg might be involved in establishing epithelial cell polarity came from few studies reporting its association with cytoskeleton (protein 4.1.) and structural (E-cadherine and β-catenine) proteins whose role in adhesion junctions formation was well documented (Hanada et al., 2003; Matsumine et al., 1996; Reuver and Garner, 1998). Direct role of Dlg in the adherens junction establishment was then demonstrated as the colonal epithelial cells in which Dlg was absent showed a marked loss of AJ integrity and failed to recruit other proteins necessary for AJ formation (p85/PI3K) (Laprise et al., 2004). This observation was later confirmed as Dlgh1 down-regulation causes junction complexes misdistribution in epithelial Caco-2 cells (Stucke et al., 2007). Dlg was found to be co-localizing with hScrib in subapical region of the mammalian epithelial cells (Dow et al., 2003); however the functional interaction between these two proteins is still lacking. Its role as tumor-suppressor seems to be intimately linked with a role of another tumor-suppressor, Adenomotous Polyposis Coli protein (APC), a protein whose mutations are involved in familial adenomotous polyposis (FAP) colon diseases and sporadical colon tumors (Groden et al., 1991; Kinzler et al., 1991). The association between Dlg and APC was soon demonstrated to negatively regulate epithelial cell proliferation (Makino et al., 1997) most likely by directly blocking G0/G1 to S phase transition (Ishidate et al., 2000). The association between Dlg and tumor development is mostly linked to its feature of being a direct target of oncogenic human papiloma viruses (HPV16 and HPV18) (Thomas et al., 2008); however the mutations in Dlg were also observed to be directly responsible for the development of tumorogenic phenotype in various mammalian cells (Humbert et al., 2008).

Lgl. The “mammalian story” of this last remaining member of Scrib family starts with a discovery of its two mammalian homologues: Lgl and Lgl2 (Wirtz-Peitz and Knoblich, 2006). However, its categorization within Scrib protein family is somewhat obscure as its colocalization with Dlg and Scrib, as well as its function in establishing/maintaining basolateral domain of epithelial cells certainly do categorize Lgl within Scrib family (Bilder et al., 2000; Bilder and Perrimon, 2000; Dow et al., 2003; Kallay et al., 2006). However, no functional link between these proteins has yet been found while, on the other side, its function is intimately related to its association with the members of Par complex, aPKC and Par3 (Yamanaka et al., 2006; Yamanaka et al., 2003). Moreover, Lgl has been shown to influence the formation of the basolateral pattern of epithelial cells by being directly involved in exocytosis *via* its association with Stx4 (a member of SNARE family) (Musch et al., 2002).

In short, Yamanaka *et al.,* proposed that Lgl is retained within the region of the basolateral membrane through its phosphorilation by aPKC, as Lgl was mislocalized in the cells where

aPKC kinase-deficient mutant (aPKC kn) was overexpressed. Since aPKC is a member of another polarity complex (Par), and since Lgl dissociates from Par6/aPKC complex upon its phosphorilation these authors suggested that the proper apico-basal polarity of epithelial cells is the consequence of the interplay between different polarity complexes (Yamanaka et al., 2003). This idea was further strengthen by the observation that apical protein disassembly was either attenuated or enhanced by interchangeable using siRNA for either Lgl or Par3, respectively. With yet another result that depletion of Lgl increases the bond between the Par6/aPKC and Par3, it might be concluded that Lgl inhibits the apical domain formation by inducing the disassembly of Par6/Par3/aPKC complex (Yamanaka et al., 2006).

Moreover, Musch *et al.* were able to see that Lgl associates with Stx4 in MDCK cells. As Stx4 is a SNARE that regulates fusion of the vesicles with plasma membrane these authors suggested that Lgl ensures apico-basal polarity of the epithelial cells by regulating secretion within basolateral membrane domain (Musch et al., 2002). These observations are not so unexpected as the same kind of functional association between Lgl and SNAREs was found also in mammalian neuronal cells (Fujita et al., 1998) and the involvement of Lgl in exocytosis was later proven in yeast as well (Zhang et al., 2005). The mechanism by which Lgl clusters Stx4-marked vesicles toward basolateral part of the membrane seems to involve cytoskeleton protein myosin, both in yeast (Rossi and Brennwald, 2011) and in mammals (Musch et al., 2002).

General mechanism of action of Scrib complex Even though the functional association between proteins of other polarity complexes (Crb and Par) has been fairly well elucidated, biochemical and functional connection between the members of Scrib family is still lacking. Their interaction has been genetically mapped (Bilder, 2003; Johnson and Wodarz, 2003; Tanentzapf and Tepass, 2003) and some of them are found physically associated in epithelial tissues (Kallay et al., 2006; Mathew et al., 2002), but no clear mechanism of action has been proposed. The role of these proteins is correlated to establishment of basal polarity probably by counteracting the function of apically situated Par and Crb complexes. Specifically, apical zone overgrowth was noticed in epithelial cells in which Crb3 was overexpressed (Roh et al., 2003) while Par3 and aPKCλ depletion induce faster disassembly of apical protein complexes. Lgl depletion in epithelial cells enhances the formation of the apical membrane domain probably by strengthening the interaction between Par6-aPKC and Par3 complex (Yamanaka et al., 2006) (Figure 4).

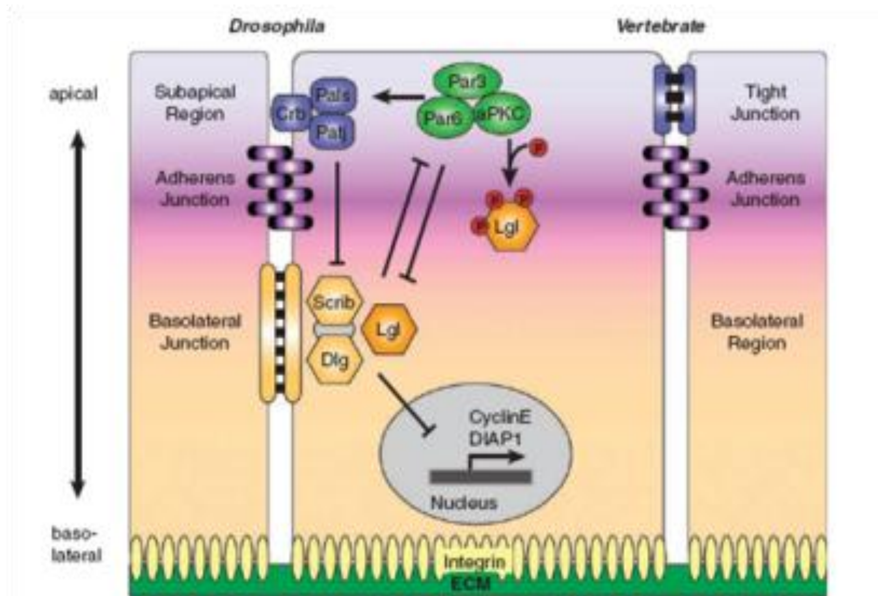

Figure 4. Polarity proteins shape apico-basal polarity of epithelial cells. Par and Crumb complex proteins are situated in the upper part of the epithelial cells where they ensure apical membrane integrity and contribute to the formation of adherens junction. Localized basally within the epithelial cells are Scrib complex proteins. Even though genetical and, to some extent, physical interaction between Scrib members has been found; biochemical and functional link is still lacking. It is thought they ensure the formation of basal domain indirectly, by counteracting the functions of Par and Crb complexes. Overall, formation of apico-basal polarity of epithelial cells is the outcome of spatio-temporal interplay between three polarity protein complexes (Humbert et al., 2008).

1.3. Regulation of polarization

Cell division cycle protein 42 (Cdc42)

So far, we have been able to see that the process of polarization within various cells involves multiple structures, mechanisms and players. However, in order to ensure proper establishment of polarization all these processes need to be spatially and temporally coordinated. Numerous research performed on this topic in various cell types revealed that cell division cycle 42 (*Cdc42 GTPase*), a GTP binding protein, is responsible for orchestrating the interplay between various polarity modules (Etienne-Manneville, 2004).

Cdc42 belongs to a family of Rho GTPases and expresses general mode of functioning shared by all GTPases: it has the capacity to bind and hydrolyze GTP, thus oscillating between GTP-bound (*active state*) and GDP-bound (*inactive state*). When in its active state, Cdc42 has the ability to pass the activation signal on its targets. In addition, three types of factors are catalyzing the GTPases activity: i) Guanine nucleotide exchange factors (*GEFs*) help GDP to GTP nucleotide exchange ii)

GTPase activating proteins (*GAPs*) catalyze the hydrolytic reaction and iii) Guanine nucleotide exchange inhibitors (*GDIs*) sequester the inactive GTPases from the membrane.

The specific property that makes Cdc42 a target of such intense research is that it directly or indirectly influences multitude of polarity-related processes in various cell types. This small molecule appears to be a main dispatcher in the cell, integrating the incoming polarity cues and translating them into organized reaction of various polarity modules. Cdc42 has been implicated in organization of a polarized phenotype of migrating cells in couple of ways. It indirectly induces actin polimerization toward the leading edge *via* WASp and p65PAK activation. Furthermore, Cdc42 exhibits a couple of integrated functions that result in MTOC reorientation toward one pole of the migrating cell. Specifically, Cdc42 stabilizes MTs through p65PAK activation and it ensures membrane docking of MTs plus end *via* IQGAP1-mediated pathway. Microtubule network stabilization and docking at the plasma membrane is thought to induce MTOC reorientation (Etienne-Manneville, 2004). Additionally, in migrating astrocytes it has been recently demonstrated that Cdc42 can directly induce MTOC polarization toward the leading edge through Par6-aPKCζ-mediated pathway. Downstream effects of this pathway induce Dlg and APC clustering at the leading edge that is further responsible for MTOC polarization (Etienne-Manneville, 2005) During epithelial morphogenesis, Cdc42 initiates the processes of polarity proteins recruitment toward junction complexes by directly binding to Par6 and inducing phosphorilation of aPKC (Joberty et al., 2000; Lin et al., 2000). In the cells of the immune system, Cdc42-dependent pathways induce actin clustering underneath the synapse area in T cells (Valitutti et al., 1995; Vicente-Manzanares and Sanchez-Madrid, 2004) and simultaneous MTOC translocation toward cSMAC zone (Banerjee et al., 2007; Stowers et al., 1995). In *C.elegans* embryogenesis, this Rho GTPase ensures asymmetrical cell division by directly affecting spindle positioning (Gotta et al., 2001). Furthermore, a few studies implicate role of Cdc42 in cell secretion as this Rho GTPase was found to regulate secretory transit between ER and Golgi complex (Wu et al., 2000) and directional secretion in epithelial cells as well (Musch et al., 2002).

Wiskott-Aldrich Syndrome protein (WASp)

Wiskott - Aldrich syndrome (WAS) is an X-linked immunodeficiency manifested in three clinical symptoms: eczema, thrombocytopenia and repeating infections. This immunodeficiency arises

as a consequence of one of few hundred possible mutations in Wiskott - Aldrich syndrome protein (WASp) occurring in the cells of hematopoietic lineage (Orange et al., 2004).

WASp contains three domains, most of them responsible for binding with various components of cytoskeleton (actin and tubulin) of with Rho GTPases (Cdc42 and Rac). However, it should be noted that WASp also contains the regions that enable it to bind to specific members of the signaling pathway (*e.g.* Fyn, Lyn Btk) as well. In homeostasis WASp is usually held in an autoinhibited status: its central (GBD) and carboxyl (VCA) domains physically interact and thus disable binding of other factors. Upon activation signal (phosphorilation), WASp is freed from the inhibited state and able to react with other factor (Cory et al., 2003; Orange et al., 2004).

The role of WASp has been mainly linked to actin cytoskeleton. Indeed, WASp was demonstrated to bind to actin either directly or by inducing the activation of Arp2/3 (Miki and Takenawa, 1998; Thrasher, 2002). WASp-mediated actin polimerization and redistribution toward cellular periphery was demonstrated to be essential for migration of various cells (Machesky and Insall, 1999; Thrasher, 2002). Furthermore, the role of WASp has been implicated in actin cytoskeleton clustering underneath the IS in the cells of the immune system (Zhang et al., 1999). The mechanism of WASp-dependent actin clustering was suggested to occur *via* activation of Zap70/CrkL/WIP/WASp pathway (Sasahara et al., 2002). Finally, WASp was found to be a link for a physical connection between actin reorganization and MTOC translocation toward the synapse in NK cells. This was verified by the absence of MTOC polarization in WASp-depleted NK cells (Banerjee et al., 2007).

1.4. Cytoskeleton and polarity proteins in immune system

Immune system is specialized to protect the body from the numerous pathogens in the environment. This implies orchestration of processes occurring at distant places and different times. In its most drastic and simplistic sense it involves pathogen recognition at the periphery and the recruitment of the multiple effector mechanisms to the site of infection *via* activation of a centralized adaptive immune response. The cells of the immune system change their location driven by soluble mediators and cytoskeleton rearrangements and ensure the transmission of the signal by engaging in highly dynamical interactions; thus overcoming this temporal and spatial gap.

1.4.1. Cell migration

1.4.1.1. The role of cytoskeleton in T cells migration

The molecular details of cytoskeleton remodeling that occurs in T cells during migration have been quite well characterized. The initial recognition of chemokine gradient at the leading edge of a migrating T cell is indirectly translated into activation of Cdc42 GTPase, *via* integrin conformational shift. This GTPase further induces actin clustering at the leading edge of lymphocytes, most likely through WASp-dependent activation of Arp2/3 complex. Actin clustering at the leading edge is important as it enables the formation of chemokine receptor-rich membrane protrusions (*i.e.* lamelopodia and filopodia) that survey the environment through their constant elongation and shrinking Mechanical forces that enable these movements are polimerization of actin on one side (*anterograde movement*) balanced by a myosin-induced backward actin movement (*retrograde movement*). Once the decision regarding directionality is made, the cell migrates by actin-based pulling onto transient structures forming between the base of the cell and the substratum (*focal contacts*) (Hall, 1998; Krummel and Macara, 2006; Rodriguez et al., 2003).

At the same time, formation of another functional microdomain occurs at the uropod of the cell. Specifically, Rho activation at the rear end leads to the activation of ezrin-radixin-meosin (*ERM*) complex, a major scaffolding protein complex that further induces clustering of other proteins Some of these proteins are the transmembrane proteins (CD44, CD44) and polarity proteins of Scrib family (del Pozo et al., 1998; Lue et al., 1996; Serrador et al., 1998). Another structure that is invariably found at the uropod of migrating leukocytes is MTOC (Affolter and Weijer, 2005; Russell, 2008). Although MTOC represents a hallmark of uropod in T cells, the mechanisms that induce its polarization are not well understood. It is, however, likely that members of Rho GTPase family are involved as their role in stabilizing/docking MT plus end and inducing MTOC translocation has been shown in various cell types (Daub et al., 2001; Manneville and Etienne-Manneville, 2006; Palazzo et al., 2004). In migrating astrocytes, MTOC polarization was shown to occur through Dlg mediated pathway (Manneville et al., 2010). Furthermore, it was suggested that MTOC can bind to two proteins localized at the uropod, myosin and meosin, and this association might be a mechanism of MTOC retention (Gundersen et al., 2004).

Functional outcome of MTOC translocation toward the rear end of migrating leukocytes is still elusive. As Golgi is invariably associated to MTOC (Kupfer et al., 1985), MTOC polarization

was suggested to have a role in directional secretion of vesicles, most likely along the axis of migration. An indication that preferential secretion is parallel to the direction of migration comes from the observation that t-SNAREs are mainly localized at cell's front. (Krummel and Macara, 2006) Microtubules have already been shown to be the major pathway for the vesicle transport in epithelial cells (Manneville et al., 2003) and they undergo post-transcriptional modifications that most likely further strengthen the binding of the vesicles (Hammond et al., 2008; Kirschner and Mitchison, 1986). Alternatively, MTOC positioning at one pole might represent a general mechanism that establishes directionality of the movement (Kodama et al., 2004) (Figire 5).

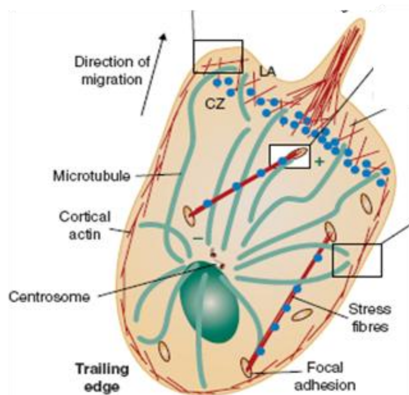

Figure 5. Organization of actin and microtubule cytoskeleton during T cell migration. In motile T lymphocytes, actin cytoskeleton is present subcortically (in order to support motile morphology of cell) and at the leading edge (where it is important for the formation of lemellopodia and filopodia). Invariably found at the uropod of motile T cells is centrosome (MTOC). Although it represents a hallmark of the uropod, its exact function is still elusive. It was suggested to have a role in establishing directionality of the movement and/or polarized vesicle secretion (Rodriguez et al., 2003).

1.4.1.2. The role of polarity proteins in T cells migration

In a study where the distribution of polarity proteins was fluorescently tracked in migrating T cells, Ludford-Menting *et al.,* were able to assess relative position of polarity proteins belonging to different complexes. In these experimental settings, they observed that Dlg and Scrib were clustered at the uropod, Crumbs3 was found distributed throughout the cell but with a slight level of overlapping with Dlg at the uropod, Par3 was localized at cell body while PKCζ did not show a clear pattern of clustering but was rather found within the whole cell. The initial clustering of Scribble at the uropod was further reflected on the assembly of other cell proteins in uropod: Ezrin, Dlg, CD44 and CD46. Finally, the loss-of-function experiments demonstrated that Scrib has a role in enabling adequate T cell locomotion, most likely due to its aggregation at the uropod (Ludford-Menting et al., 2005). Proteins belonging to Par complex have also been implicated in ensuring migration capacity of the cells of the immune system. Par3 and PKCζ enable proper T cell migration by chemokine induced clustering at the leading edge (Gerard et al., 2007). In line with that is the observation that clustering of PAR6 and

aPKC at the leading edge of migrating neutrophilles is involved in the migration of these cells (Macara, 2004). The role of Crumbs complex in migratory capacity of the cells has been very poorly understood (Etienne-Manneville, 2008), while the implication this complex might have on leukocyte migratory capacity has not been tackled so far. However, while Dlg and Scrib are invariably found located at the uropod of the cell (Cullinan et al., 2002; Krummel and Macara, 2006), the exact location of Par protein family is still a matter of controversy. For instance, the involvement of aPKC in lymphocyte migration by their clustering at uropod has also been documented, as the expression of dominant negative aPKC induced uropod malformation and F-actin perturbation. Although to a lesser extent, similar phenotype of uropod disappearance was seen in cells expressing Par6 dominant negative mutant, suggesting that these two proteins might be working in the same pathway in T cell locomotion (Real et al., 2007). The localization of Lgl was suggested to be mainly within T cell midbody (Krummel and Macara, 2006). The localization of Lgl outside of its corresponding complex (Scrib complex) is not that surprising, as its functional association with a protein from other polarity complexes (aPKC) has been well established (Yamanaka et al., 2006). Finally, the regulators and mechanisms necessary to coordinate polarity proteins assembly in the context of leukocyte movement have been very poorly understood, but a few reports state the action of Cdc42 in initial clustering of Par complex proteins as a prerequisite of this process (Gerard et al., 2007; Macara, 2004) (Figure 6).

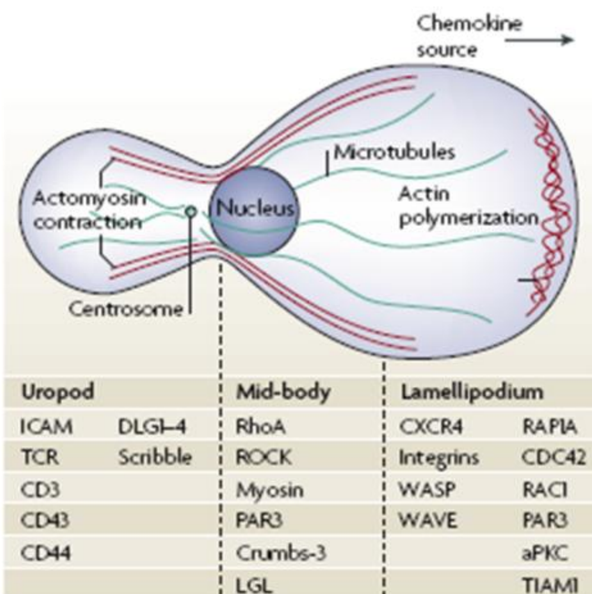

Figure 6. A model representing relative distribution of polarity proteins in migrating T cell. Although the exact positions of polarity proteins within motile T cell are still elusive, the data coming from few initial studies have helped establish a general model. Par polarity complex proteins are mainly localized at the leading edge, although some Par3 may also be found in the midbody. Crb proteins are nestled at the midbody, while Scrib proteins (Scrib and Dlg) are invariably found at the uropod. Lgl, however, is found in the midbody; this is not surprising as its functional and physical association with members of other polarity protein complexes has already been documented (Iden and Collard, 2008).

1.4.1.3. DCs migration

General picture of DCs migration suggests that migration capacity of DCs is mirrored in the formation of actin cytoskeleton-based specific structures: filopodia, lamelopodia and podosomes. In migrating cells, filopodia and lamelopodia are the chemokine receptors rich structures forming at cells' anterior that, through constant elongation and shrinking, enable the cell to sense the directionality of chemokine gradient. Podosomes are a network of actin-based adhesive spots, predominately found behind the leading edge of monocyte-derived cell (in DCs), that enable cell movement by transient attachment and disattachement from the substratum, through integrin-mediated processes (Burns et al., 2001; Linder and Aepfelbacher, 2003; Rodriguez et al., 2003). Proteins vinculin, paxilin and talin are also hallmarks of these structures and are essential to link integrin binding to F-actin polimerization thus enabling the fluidity of the movement. The stability of cellular migration is ensured by the formation of a thin protrusion, called uropod, at the rear end of the cell. The membrane of uropod is filled with the receptors for integrins (*i.e.* ICAM-1, ICAM-3) which, through binding to corresponding integrins expressed on the endothelial cells, transiently "lock" the cell at one position. The change in chemokine gradient perceived at the anterior part of the cells induces uropod displacement in the same direction, through active remodeling of actin cytoskeleton.

Kinetics of these DCs structures is timely regulated by coordinated activation of small Rho GTPase: Rho, Rac and Cdc42. Specifically, by independently microinjecting dominant negative or constitutively active forms of each of these three Rho GTPases, Burns *et al.* were able to follow the effect they have on the migratory structures formation and DCs migration in general. Thus, these authors report that all three GTPases are important for the formation of podosomes while Cdc42 has additional roles in ensuring filopodia directionality and establishing DCs polarity phenotype in general (Burns et al., 2001). The functional role of Rac1 and Rac2 in migration capacity was demonstrated by defective *in vitro* and in *vivo* migration of DCs lacking these proteins that further jeopardized DCs capacity to adequately prime naïve T cells (Benvenuti et al., 2004a).

Most of the migration characteristics of DCs have been shown to rely upon WASp-mediated Arp2/3 polarization of actin cytoskeleton. This protein has been shown to be enriched at the podosomes region of migrating cell. The cells lacking WASp failed to cluster Arp2/3 at the leading edge which induced a block in actin polimerization and subsequent deformities in protrusive structures. Furthermore, the cells that lacked WASp were unable to propagate the leading edge and had difficulties

to disattach the uropod. This reflected on their inability to migrate *in vitro* and *in vivo* (Burns et al., 2001; Calle et al., 2004; de Noronha et al., 2005).

Important to say, however, is that recent technological advances, enabling observation of leukocytes (and DCs) movement in 3D matrix, propose that the necessity for integrin mediated podosomes formation as the means for DCs migration is a reflection of 2D environment limitations) (Friedl et al., 1998; Pierre, 1997). Instead, they suggest that a sheer flow of actin network growth at the leading edge is the exclusive cause of DCs migration. The contractile myosin force at the uropod of the cell is necessary only to enable nucleus "squeeze" through ECM. During this movement, the cells acquire a more rounded, flexible phenotype characterized by a loss of any adhesive properties and podosoms structures. The "replacability" of integrins for leukocyte migration was verified by the maintenance of normal motile movement in cells in which all integrin heterodimers were depleted (Lammermann et al., 2008; Lammermann et al., 2009). Furthermore, the *in vivo,* but not *in vitro* migration, of DCs was completely abrogated in cells lacking Cdc42. Specifically, these cells retained the capacity to polimerize actin, they generated contractile actomyosin forces, the formation of protrusions was not jeopardized and the cells maintained normal migratory capacity in 2D. However, the coordination of protrusions was spatially and temporally disorganized which further caused cells entanglement in 3D matrix. This suggested that DCs movement is the consequence of Cdc42-dependent temporal and spatial organization of actin filaments rather than any kind of attachment with substratum (ECM) (Lammermann et al., 2009). Finally, the possibility that DCs might follow both integrin-dependent and integrin-independent migration patterns was also suggested. Specifically, depletion of cytohesin-1 (a guanine-exchange factor) abrogated DCs movement *in vitro* and *in vivo*, suggesting its role in DCs motility. However, whilst its depletion did inhibit the movement of integrin-depleted DCs in 3D matrix, it did not affect the corresponding movement of DCs where integrins were preserved (Quast et al., 2009).

1.4.2. Immunological synapse (IS) formation

Immunological synapse (*IS*) is specialized structure formed at DC-T cell interface where transmission of the signals regarding the nature of the pathogen is exchanged. Initial MHC-TCR signaling induces drastic morphological changes of the cells: they "abandon" their migration phenotype and acquire more rounded, stable shape. The axis of symmetry is shifted from anterior-posterior one to

apico-basal one. All this is achieved by a global redistribution of cytoskeleton elements and formation of transient polarity proteins modules (Negulescu et al., 1996).

1.4.2.1. The role of cytoskeleton in T cells during IS

Upon TCR signaling, actin cytoskeleton, clustered subcortically and tied to the membrane in migrating cells, now liberates itself from membrane bonds and polarizes toward synapse region. This is thought to occur through three events following TCR activation. Phosphorilation of ERM complex at uropod, responsible for retaining actin bound to the membrane, induces a conformational shift in this complex which now assumes a more closed conformation, unable to bind to actin. At the same time, myosin phosphorilation is thought to induce a release of actin filaments in leading edge and body of a migrating cell. The third mechanism that gets activated upon TCR binding is WASp-mediated Arp2/3 activation, a mechanism that leads to actin polarization beneath synapse region (Barda-Saad et al., 2005; Dustin and Cooper, 2000; Faure et al., 2004; Jacobelli et al., 2004). This was supported by a reduced capacity of WASp knockout T cells to polimerize and cluster actin at IS in response to CD3 triggering (Gallego et al., 1997). Upstream signaling cascade involved in WASp activation incorporates Cdc42-mediated formation of a transient signaling complex-Lck/Zap70/Dlg/WASp (Cannon and Burkhardt, 2002; Round et al., 2005; Zeng et al., 2003). All this finally results in actin polimerization and its polarization within pSMAC region of the synaptic cleft. The network of clustered actin sheets secures a strong cellular interaction as the inhibition of actin polimerization by cytohalasin D affects IS stabilization (Delon et al., 1998; Valitutti et al., 1995).

At present, in many cells of the immune system, the phenomenon of MTOC polarization toward synapse region is very well established. The initial data, however, regarding this phenomenon came from the pioneering works of Kupfer *et al.* where these authors demonstrated that MTOC polarization occurs in cloned NK cells toward a synapse with a target cell (Kupfer et al., 1983) as well as in T cells (Kupfer et al., 1987). Further experiments in this field strengthen these findings and further elucidated the machinery and signaling cascades integrated in this process. Visualization of the phenomenon of MTOC translocation (*via* modulated polarization microscopy (MPM) and 3D reconstruction) offered better insight into the nature of MTOC movement: MTOC was visualized to go vertically toward the synapse following the sliding MT movement and finally localizes beneath cSMAC zone (Kuhn and Poenie, 2002). The phenomenon of MTOC translocation in T cells following TCR

activation was shown to relay on dynein/dynactin activity (Martin-Cofreces et al., 2008). Moreover, MTOC polarization was reasoned to involve the activation of Zap-70, LAT and Vav1, as dynein/dinactin depletion did not only inhibit MTOC translocation but also reduced phosphorilation of these downstream signaling players. Stowers *et al.* have undoubtedly showed Cdc42 is essential for MTOC polarization toward the synapse in T cells as the expression of both constitutively active and dominant negative mutant of Cdc42 inhibited MTOC polarization (Stowers et al., 1995). Downstream mechanisms engaged in MTOC polarization might involve Cdc-42 interacting protein 4 (CIP4) and ERK2 pathway as demonstrated in NK cells (Banerjee et al., 2007; Chen et al., 2006).

The initial reports suggesting the possibility that polarized clustering of intracellular components on one side of the synapse might have an impact on destiny of the cells on the other side of the synapse came from the works of Kupfer *et.al.* In short, the authors were able to see that polarized movement of MTOC and Golgi complex in cytotoxic T lymphocytes is necessary for the lysis of bound cells (Kupfer and Dennert, 1984; Kupfer et al., 1985). Moreover, by using a model system of T cell forming multiple synapses with B cells, they were able to see that only B cell toward which MTOC translocation occurred underwent mitosis. On the contrary, the proliferative cycle of the other B cell was stopped at the interphase (Kupfer et al., 1994). What further strengthens this hypothesis was the finding that MTOC is one of the prominent players in ensuring the asymmetrical division of the T cells upon their prolonged contact with APC (Chang et al., 2007). Another suggestion that MTOC polarization might be connected with a delivery of a functional message was demonstrated by the observation that NK cells in which MTOC polarization was abrogated failed to induce target cell death (Chen et al., 2006) (Figure 7).

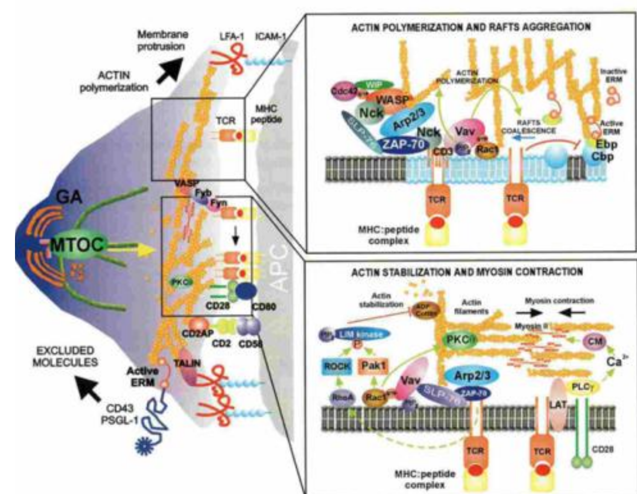

Figure 7. Actin and MTOC position during IS formation. During synapse formation, actin cytoskeleton clusters at the zone beneath the IS. The process of actin clustering occurs as a result of ERM and myosin phosphorilation and WASp activated Arp2/3 nucleation. The role of actin is supportive, as inhibiting actin polimerization strikingly affects IS stability. Following TCR stimulation, MTOC moves in close proximity to IS. Its reorientation toward the synapse region is ensured by the activation of small Rho GTPase (Cdc42) and probably pulled by dynein/dynactin forces. Numerous reports state the role of MTOC as a general scaffolding structure necessary to deliver lytic granules toward synapse both in CTL and NK cells.

1.4.2.2. The role of polarity proteins in T cell during IS

A fair number of reports state that Dlg and Scribble are important for the formation and maintenance of the immunological synapse. More specific, in the experiments with antibody-conjugated beads and antigen-loaded B cells, Xavier R *et.al.* have shown that thus stimulated T cells show a transient polarization of Dlg1 toward IS. Upon polarization Dlg1 associates with signaling molecules present at the synapse region (TCRζ and Cbl), while a certain portion of Dlg undergoes Lck-dependent phosphorilation (Hanada et al., 1997; Xavier et al., 2004). In similar report, Ludford-Menting *et.al.* further elucidate the causal interactions of these proteins by showing that Dlg, Scrible and ezrin transiently co-localize at the synapse as the consequence of TCR signaling. Moreover, fluorescent tracking of two other polarity proteins, Par3 and Crumbs3, revealed that they also undergo dynamical redistribution relative to the synapse with DCs, but following the patterns completely opposite from Dlg and Scrib (Ludford-Menting et al., 2005). This is homologues to their mutually exclusive distribution during the formation of apico-basal asymmetry in epithelial cells (Bilder et al., 2000), as well during T cell migration (Krummel and Macara, 2006). In the final experiment with anti-CD3 and anti-CD28 coated beads, it was shown that Scribble is important for the synapse functioning as knockout T cells show a striking defect in their ability to form the stable conjugates (Ludford-Menting et al., 2005). The functional outcomes of Dlgh1 polarization toward synapse post TCR activation were shown to induce: TCR clustering, autocrine cytokine production (IL-2 and IFN-γ) and enhanced NFAT signaling. This action of Dlg was shown to be obtained through a formation of Lck/Dlgh1/Zap-70/WASp complex (Round et al., 2005).

However, on the other side, Dlg has also been attributed the role as a factor that expresses inhibitory effect on TCR signaling. In short, Stephenson LM *et.al.,* have demonstrated that primary Dlgh1-deficient T cells respond to TCR activation by increasing their rate of proliferation. The absence of Dlg at the synapse in these cells did not affect other synapse-specific events: the clustering of other synapse proteins (Scrib, Vav and ZAP-70), the incidence of MTOC translocation toward IS, the activation of kinase pathways (Erk1/2, Akt and p38), nor cytokine production capacity (IL-2 and IFN-γ) (Stephenson et al., 2007). Inhibitory effect of Dlg on T cell activation was also attributed to the decrease in NFAT signaling in T cells in which Dlg has been depleted (Xavier et al., 2004). Even though these results could be attributed to the tumor-suppressor capacity of Dlg, it is evident that further experiments are needed in order to clearly nail the function of Dlg during synapse formation in T cells.

Furthermore, the polarity proteins clustering at the immunological synapse in T cells have been shown to affects T cells fate determination. Specifically, in the experiments with *in vivo* induced asymmetrical T cell division, it was seen that specific proteins (Scrib, CD8 and PKCζ) segregate asymmetrically. In short, CD8 and Scrib were segregated into one daughter T cell and PKCζ was segregated into the other T cell. This later resulted in the existence of two daughter cells with phenotypical and functional (IFNγR) markers specific for either effector or memory T cell (Chang et al., 2007). From this example it is obvious that the unequal distribution of the polarity proteins prior to cell division is a crucial event in directing fate and functionality of T cells. Furthermore, by setting an *in vitro* model to follow the inheritance of different fate determinants during T cell mitosis, (while still in synapse with APC), Oliaro *et.al.,* were able to see that Dlg/Scrib and Par3/aPKC were enriched at different regions in mitotic T cells, in respect to attached APC. Dlg and Scrib were mostly localized at the proximal part of mitotic T cells, which consequently resulted that the majority of Scrib was present in future T cell proximal to the APC. On the contrary, Par3 and aPKC were mainly clustered at the distal part of the early dividing T cell, and thus within future T cell distal in respect to APC. This further induced the asymmetrical segregation of Numb, whose presence was dependent on the presence of Par3 as well as on the phosphorilation by aPKC (Oliaro et al., 2010).

There are some reports regarding mechanisms of action of Dlg within IS, however, the whole story has just started to be revealed. Specifically, Dlg has been demonstrated to affect MTOC translocation toward IS in T cells, most likely through Dlg-dependent (p38) NFAT activation (Lasserre et al., 2010). Another intriguing study, adding to the complexity of IS signaling, suggests the activation of two different activation pathways in naïve and activated T cell, both in response to TCR activation. Specifically, in naïve T cells TCR signaling induces the activation of ERK pathway, while in activated T lymphocytes TCR activation induces Dlg-dependent phosphorilation of p38. The interaction between Dlg and p38 in activated T cells was also shown to be important for IFN-γ producing capacity of these cells (Adachi and Davis, 2011).

The role of members belonging to other complexes (Par and Crb) has been very poorly elucidated. Par1b has been shown to be phosphorilated by PKCζ and clustered at the synapse following TCR signaling. This enrichment at the synapse was shown to be important for MTOC translocation, as Par1b depletion affected the rate of MTOC translocation (Lin et al., 2009). Crumbs1 was shown to be constantly present within IS region of T cells, but the mechanism or the function of this phenomenon are still not clear (Ludford-Menting et al., 2005).

1.4.2.3. DCs in synapse

Even though the contribution of actin based membranous protrusions in the formation of informative synapse has been very well documented in T cells (Dustin and Cooper, 2000) until recently it was thought that APCs (DCs) represent only an immobile surface, a platform that bestows stability to IS and thus enables the passive transmission of the signal to T cells (Valitutti et al., 1995). However, the array of reports has negated this paradigm demonstrating a very active DCs involvement in initial contact with naïve T and in IS preservation.

Specifically, DCs actively polarize cytoskeleton (fascin and F-actin) during interaction with T cells. This was shown to have marked impact on the formation of DC-T cell conjugates, as well as on the activation of T cells (Al-Alwan et al., 2003; Al-Alwan et al., 2001b). The mechnism of actin polarization in DCs during synapse formation was further deepened as it was shown to occur only in antigen (MHC) specific manner (Boes and Ploegh, 2004). The role of WASp as the organizer of actin cytoskeleton during IS formation was also demonstrated as DCs lacking WASp had an impairment in actin cytoskeleton polimerization and failed to form stable synapses with T cells *in vivo* and *in vitro* (Bouma et al., 2007; Pulecio et al., 2008).

By using time-lapse video microscopy Benvenuti *et al.* were able to follow and give initial insight into the kinetics of DC-T cells interactions *in vitro* and *in vivo.* These authors were able to see that DCs morphology is characterized by constant extension and retraction of symmetrical short, spike-like membranous structures, called dendrites. The initial contact with a T cell induces asymmetrical growing of dendrites on the side facing T cell, followed by DCs body displacement and complete “drowning” of T cells within these protrusions (Benvenuti et al., 2004a). These protrusions most likely represent a structural prerequisite of the IS as they enhance the contact area between DC and T cell, while the acquirement of the DCs “mature” phenotype is actually what induces the activation of T lymphocyte (Benvenuti et al., 2004b). IS formation is a function of the events occurring during DCs maturation as only these cells formed durable contacts with T cell. On the contrary, immature DCs established a series of short, intermittent contacts with T cells not sufficient for T cell activation. Furthermore, only in the stable synapses the clustering of transmembrane molecules (LAT, LFA-1 and CD3) within SMAC region at DC-T cell interface was visible (Benvenuti et al., 2004a; Benvenuti et al., 2004b). The engagement of integrin (LFA) molecules on DCs side by ICAM-1 and ICAM-3 on T cells

side (clustered within SMAC region following the TCR contact) was shown to activate Vav-1 and Rac1 pathway in DCs and induce even higher expression of MHC molecule (de la Fuente et al., 2005).

Another piece of evidence demonstrating that DCs actively contribute to the formation of synapse comes from the studies where two-photon microscopy was utilized. These studiers enabled the visualization of DC-T cell contact *in vivo* and revealed the complexity and dynamics of the processes. A lymph node is a very lively environment where naïve T cells demonstrate extremely high random movement while DCs exhibit by far less locomotion; they are rather localized in strategic position (proximal to HEV), constantly probing the environment through rapid dendrites motion. It is actually this diversity in T cell and DCs behavior the thing that optimizes the encounters between the cells and enables the transmission of the information. The complexity of the DC-T cell crosstalk is mirrored even further as, once engaged, DC and T cells were shown to establish very diverse types of contacts: from short, transient interactions *via* stable, long-lasting ones to the establishment of brief contacts between one DC and multiple T cells (referred as *swarms*) (Bousso, 2008). It is still not clear what dictates the transition from the phase of initial contacts to the formation of long-lasting contacts; however, it was suggested to be a function of the time of interaction and/or the antigen dose presented (Henrickson et al., 2008; Mempel et al., 2004). A recent study, performed by Scholer *et al,* suggests a crucial role of adhesion molecules in determining the duration of the contact and T cell fate. Specifically, these authores noticed that DCs upregualte ICAM-1 following TCR signaling and that ICAM-1 deficient DCs established only short interactions with T cells and failed to induce IFN-γ production in T cells (Scholer et al., 2008). Furthermore, it is becoming ever more evident that DCs maturation status and dynamical changes occurring in DCs are directlly responsible for the induction of immune response or tolerance (Hugues et al., 2004).

Although intracellular signaling pathways extending from IS to DCs are still obscure, the activation of Rho GTPase within these pathways has been very well documented. Specifically, Rac1 and 2 in DCs have been shown important for the activation of long lasting synapses with T cells (Benvenuti et al., 2004a). Rho GTPase is important to secure actin polimerization and formation of dendrites in DCs, which enables interaction with CD4+ T cells and their activation (Kobayashi et al., 2001). It is also possible that proper activation of T cells by DCs is a result of interplay between three GTPases (Cdc42, Rac1 and RhoA) (Shurin et al., 2005). Furthermore, recent reports indicate that IS formation transmits pro-survival signals to the engaged DCs as they upregulate the expression of anti-apoptotic genes which most likely further stabilizes IS (Riol-Blanco et al., 2009; Rodriguez-Fernandez et al., 2010). The initial

reports that tackled the localization of polarity proteins in DCs during synapse formation indicated that spinophillin and anexin-2 are found enriched at the synapse region in DCs and suggested their role in IS signaling (Bloom et al., 2008; Eun et al., 2006).

1.4.2.4. Polarized secretion

Factors secreted by lymphocytes are often released into the environment that is densely populated with many cell types, which brings the problem of specificity of intercellular communication The observations that MTOC and polarity proteins polarization toward the synapse have an impact on the fate of the bound cells suggested a possibility that certain factors secreted exclusively within IS might be responsible for maintenance of IS integrity and specificity.

In an extensive study employing live-cell imaging and surface-mediated secretion assay in order to follow trafficking and secretion of various cytokines in Th cells in synapse with APC, Huse *et.al.,* have clearly demonstrated that these soluble mediators can be secreted within two different pathways, what they termed: directional and multidirectional secretion. More specifically, they were able to see that IL-2 and IFN-γ are secreted in synapse area, while TNFα and CCL3 (Mip-1α) followed a more dispersed pattern of secretion (Huse et al., 2006). A possibility of polarized secretion of cytokines was suggested even earlier by Kupfer A *et al.,* who noticed that IL-2 and IFN-γ in Th cell (and IL-4 and IL5 in the other T cell clone) were clustered close to the synapse area with B cell in antigen-dependant manner (Kupfer et al., 1991). An indication that polarized secretion of cytokines might have a physiological significance comes from the immunohistological studies that demonstrated that IL-2 preferentially localizes in synapse area of CD4+T cells (in mice injected with antigens) (Reichert et al., 2001). A few reports that tackled the question of polarized secretion in DCs clearly suggested that the formation of informational synapse relies on the events occurring simultaneously on both side of the synapse. Specifically, DCs in the synapse with NK cells undergo the changes in their cytoskeleton and release IL-18 rich vesicles toward NK. Simultaneously, NK cells release a pro-inflammatory cytokine (HMGB1) that induces DCs maturation and enhances their survival (Semino et al., 2005). Synapse formation between NK and DCs triggers polarization and secretion of IL-12 by DCs toward bound NK (Borg et al., 2004).

Following studies aimed to further elucidate the phenomenon of polarized secretion, revealed the complexity of the structures and delineated other players involved in this process. By

employing fluorescence staining and electron microscopy (EM) techniques Stinchcombe *et al.,* were able to observe that membrane region within IS is not a plain, amorphic structure but is rather composed of well-defined functional domains. Specifically, immune synapse in CTL was shown to contain the adhesion ring (where the cytoskeleton clustering occurs), signaling ring (the place of receptor binding) separated by a domain where cytokine secretion specifically occurs (secretion domain) (Stinchcombe et al., 2001b). Additionally, numerous reports state the role of MTOC as a general scaffolding structure necessary to deliver lytic granules toward synapse both in CTL and NK cells (Banerjee et al., 2007; Chen et al., 2006; Jenkins et al., 2009; Stinchcombe et al., 2006). The concept of MTOC translocation has found its implication in ensuring proper antigen-processing and presenting capacity of B cells, since the delivery of MHC-rich lysosomes in order to perform pH-dependent capturing of immobilized antigens was shown to be MTOC dependent. Another appealing observation coming from this study was that the process of MTOC translocation was regulated by Cdc42-dependent activation of aPKCζ (Yuseff et al., 2011).

These discoveries clearly implied that the synchronization of cellular functions is sustained not only by receptor-mediated activation of specific signaling cascades, or by temporal coordination of gene expression; but it also involves a well designed and highly regulated trafficking of responsive information. It is well established that secretion involves an organized process of vesicle budding and fusion, and transmembrane proteins SNAREs (*soluble NSF attachment protein receptor*) emerged as the molecules that have a prominent part in this process by ensuring specificity of membrane fusion events (Chen and Scheller, 2001; Jahn et al., 2003; Weber et al., 1998). An array of studies where both SNAREs and cargo were fluorescently tracked in one cell, helped reveal the complexity of intracellular trafficking pathways and preferential places of secretion in various cell types. By utilizing the fact that different vesicles in the exocytic pathway are characterized by the presence of different SNAREs on their membrane, Murray *et al.,* were able to follow trafficking and secretion pattern of TNFα in activated macrophages (Murray et al., 2005). Specifically, by fluorescently co-staining TNFα and VAMP3 (a SNARE present in the recycling endosomes (RE) membrane in macrophages), these authors observed that TNFα secretion goes through RE, as TNFα signal was highly enriched in this organelle. Furthermore, RE delivers TNFα specifically toward the zone of phagocytic cup formation, as demonstrated by high VAMP3 fluorescent signal in that area. SNAREs are responsible for the discrimination between distinct pathways of secretion of azurophillic and specific granules in neutrophilles during inflammatory reactions (Mollinedo et al., 2006). Polarized secretion of cytolitic

granules both in CTL and NK was shown to rely on Munc 13-4 and VAMP7 dependent exocytic pathways, respectively (Bossi and Griffiths, 2005; Marcet-Palacios et al., 2008). Furthermore, a highly organized secretion pathway appears to be utilized in order to target receptor toward IS. In short, by following cretion pathway of TCR in a T cell engaged in antigen-specific synapse, Das *et al.,* were able to see that TCR transits through RE while Stx4 clustering underneath the synapse represents a signal for its specific targeting toward the synapse (Das et al., 2004). Finally, the putative role of SNAREs in distinguishing between polarized secretion of cytokines toward IS or multidirectional secretion in Th cells was suggested (Huse et al., 2006).

Moreover, Rab GTPases are another group of proteins ascertaining the spatial orchestration of endo/exocytotic processes in various cells, as they ensure the initial recognition between the membranes. For instance, Rab27a is involved in regulating the secretion of lytic granules in CTL (Stinchcombe et al., 2001a) and ensures the process of antigen presentation in DCs by mediating initial contact between antigen carrying phagosomes and lysosomes-like organelles in the presentation pathway in these cells (Jancic et al., 2007). In T helper cells, Rab3d is essential to ensure initial docking of the vesicles containing cytokines (IL-2 and IFN-γ) to synapse membrane (Huse et al., 2006). These and many other studies helped reveal the existence of temporally and spatially highly organized machinery for vesicles trafficking, consisting of: vesicle transport (performed by cytoskeleton), initial docking (referred as *tethering*) of membranes (performed by Rab family) and final fusion (performed by SNAREs) (Behnia and Munro, 2005; Huse et al., 2008; Spiliotis and Nelson, 2003).

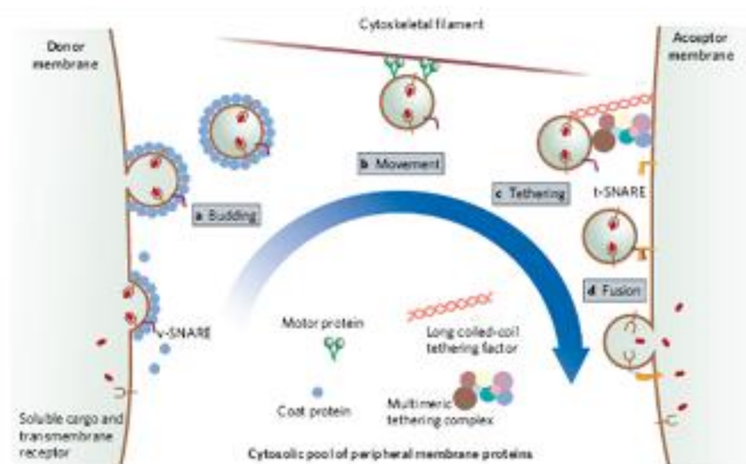

Figure 8. Schematic representation of polarized secretion. Vesicle transport within the cells includes following steps: budding, transport, tethering, and fusion. The vesicles budding from the donor membrane (*e.g.* Gogli complex) are transported toward target membrane *via* cytoskeleton network. The initial contact between the vesicle and target membrane is enabled by small Rab GTPases (*tethering*). Final step of fusion is mediated by specific recognition between specialized transmembrane proteins at vesicle (v-SNAREs) and target (t-SNAREs) membranes (Behnia and Munro, 2005).

Interleukin 12 (IL-12), an example of a third signal

Interleukin-12 (IL-12) has a function in the activation of the early innate response against intracellular microbes, by stimulating IFN-γ release and enhancing citolytic capacity of NK cells (Steinman and Hemmi, 2006). Furthermore, it is also a key factor that activates the cellular part of the adaptive immune response by enhancing the differentiation of CD4+ and CD8+ T cells toward their specific lineages (Kalinski et al., 1999a; Villadangos and Schnorrer, 2007)

IL-12 is a heterodimer, consisting of the 35-kDa (IL-12p35) and 40-kDa (IL-12p40) subunit. Many cell types have the ability to produce IL-12p35, but only the antigen-presenting cells (APC) are capable of producing both of the subunits, thus forming a biologically active heterodimer – IL-12p70 (Trinchieri, 2003). DCs release IL-12 in several "waves" during immune response as a result of interaction between various molecules and their recepotors on DCs. A few stimuli are able to induce the IL-12 release from the DCs: (i) initial recognition of pathogens by TLR (Janeway and Medzhitov, 2002) (ii) IFN-γ R engagement on DCs by IFN-γ released by NK cells during the innate immune response and T cells during the adaptive immune response (iii) signaling occurring during antigen-specific synapse with T cells. Activated T cells express CD40L, whose interaction with CD40 on DCs side induces an increase in the expression of the co-stimulatory molecules (CD80 and CD86) and upregulation of IL-12 production (Trinchieri, 2003)

IL-12 binding is a decisive step that polarizes the differentiation of the CD4+T and CD8+ lymphocytes toward Th1 and CTL lineages, respectively. IL-12 binding to IL-12R expressed on T cells is translated into a unique combination of the signal transduction molecules: JAK2 (Janus kinase) activation and STAT4 phosphorilation. Thus activated, STAT4 induces the expression of IFN-γ, a "signature" cytokine of Th1 and CTL lineages (Bacon et al., 1995a). IFN-γ then ensures a complete polarization of T cell immune response toward Th1 and CTL dominated response by enhancing the production and expression of IL-12 receptor subunit (IL-12β2R). Naturally, this represents a particular mechanism of a positive feedback loop, since it further sensitizes activated T cells to IL-12. Additionally, IFN-γ and IL-12 suppress T cell differentiation toward Th2 lineage by inhibiting the expression of GATA-3. Upon release, IFN-γ enable the elimination of pathogen by expressing numerous functions: i) augments macrophage mikrobicidal activity ii) upregulates MHC expression on APC iii) initiates IgG-dominant isotype switching in B cells and inhibits IgE isotype switching (Trinchieri, 2003).

Materials and Methods

Mice

Six to eight week old C57BL/6 females were purchased from Harlan. GFP-centrin mice were generated from a construct provided by M. Bornens (Institut Curie, Paris, France) and were a gift from C. Desdouets (Institut Cochin, Paris, France). WASp- mice of C57BL/6 (CD45.2) genetic background were a gift from S.Snapper (Massachussets General Hospital, Boston). OVA-specific, MHC class I restricted and MHC class II, TCR transgenic OT I and OT II mice were purchased from the Jackson Immuno Research Laboratories. CD45.1 congenic C57BL/6 (a gift from P. Guermonprez, Institut Curie, Paris, France) 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). Protocols were approved by the Italian Ministry of Health.

Cells

Bone marrow–derived DCs were differentiated *in vitro* from the bone marrow of C57BL/6 or centrin-GFP knockin mice using culture medium containing Fms-like tyrosine kinase 3 ligand. DCs were used for experiments between days 7 and 8, when expression of Cd11c was higher than 80%.

Western blot analysis

In order to check Dlg1 expression profile $5x10^5$ cells were stimulated with a mix of TLR agonist, CpG and LPS (10 µg/ml), for different periods. At the end of the incubation periods, the cells were washed in PBS and lysed with E1a lysis buffer (50 mmol/L HEPES [pH 7.4], 0.1% Nonidet P-40, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L NaF, 10 mmol/L) for 20min on ice. The lysate was centrifuged at 1500rpm for 2min and soluble fraction was run on 14% SDS-PAGE. Membranes were blocked in TBS, 5% raw milk, 0.02% Tween-20 and developed using rabbit anti-Dlg1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-rabbit horse radish peroxidase (Sigma-Aldrich). In order to determine the loading levels, rabbit anti-actin antibody (Sigma-Aldrich) was used.

RNA isolation, RT-PCR and qRT-PCR

Total RNA was isolated by TRI Reagent isolation kit (Sigma-Aldrich), following manufacturor's instructions. DNA was removed from isolated RNA fraction by a treatment with RNAse-free DNAse I

(Fermentas Inc, Massachussets, USA). Total RNA was retro-transcribed to cDNA by Moloney murine leukemia virus RT (M-MLV-RT) in the presence of random hexamers (IDT). Primers used for PCR amplification of Dlg1 were a kind present from N. Narayan (ICGEB, Italy). PCR products were run on 1% agarose gel and visualized on UV-transilluminator. qRT-PCR was based on SYBR Green Master Mix (Applied Byosystems) technology. The levels of target gene expression were normalized to Actin and/or GAPDH expression levels. Primers used for qRT-PCR were as follows: Dlg1 (F: GCAGGCCAGAAAAACATTTGAG-3'; R: TCTCCCTGGACAATAGCTGTGAA-3;-); IFN-γ (F: 5'-ATGAACGCTACACACTGCATC-3'; R: 5'-CCATCCTTTTGCCAGTTCCTC-3'), IL-12p35 (F: 5'-GGCATCCAGCAGCTCCTCTC-3'; R: 5'-ACCCTGGCCAAACTGAGGTG -3') , IL-12p40 (F:5'-TGGTTTGCCATCGTTTTGCTG-3'; R: 5'-ACAGGTGAGGTTCACTGTTTCT-3') GAPDH (F: 5'-AGAAGGTGGTGAAGCAGGCATC-3'; R: 5'-CGAAGGTGGAAGAGTGGGAGTTG-3') and actin (5'- GGCACC ACACCTTCTACAATG-3'; R: 5'GTGGTGGTGAAGCTGTAGCC-3'),

Imaging

To induce maturation, DCs were stimulated with a mixture of TLR agonists CpG and LPS (10 µg/ ml) for 4 and 5 h. For synapse experiments, DCs were pulsed with graded dose of the MHC class I restricted peptide of OVA 257-264 (SIINFEKL) and transferred to slides coated with fibronectin (Sigma-Aldrich; 10 µg/ml). OT-I cells were added to DCs in a 1:1 ratio and incubated at 37°C for 5, 15 and 30 min. In some experiments, OT-I were labeled with 2 µm CFSE to facilitate detection of DC–T cell doublets. Nonadherent T cells where removed by washing the slides with PBS several times. Cells were fixed (4% paraformaldehyde), permeabilized (PBS/BSA 0.1%/saponin 0.05%), and immunolabeled. The following antibodies were used: rat anti α-tubulin (AbD; Serotec), rat anti–IL-12 p40/p70 (BD), rabbit anti-Dlg1 (Santa Cruz Biotechnology, Santa Cruz, CA), hamster anti-CD3 (BD), mouse anti–VAMP-7 (provided by T. Gally, Institut Jaques Monod, Paris, France), and rabbit anti–γ-tubulin (provided by M. Bornen, UMR144, Institute Curie, Paris). All secondary antibodies were obtained from Invitrogen. Phalloidin-Texas red (Sigma-Aldrich) was used to detect polymerized F-actin. Confocal images were acquired in a LSM510 META Axiovert 200M reverse microscope with a 63x objective (Carl Zeiss, Inc.). Z projection of slices, three-dimensional reconstruction, and image analysis were performed using an LSM image examiner (Carl Zeiss, Inc.) and ImageJ software (National Institutes of Health).

Analysis of polarization

The analysis of polarization was performed on DCs conjugated to a single T cell. This is the most represented condition when a 1:1 DC/T cell ratio is used. To score conjugates with polarized MTOC or Dlg1 we calculated the ratio between the DC's diameter and the distance of the MTOC or Dlg1 to the synapse region. Conjugates in which such value was <0.3 were considered "polarized." In Fig. 3b, 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 <0.3. To evaluate whether DC polarization is linked to activation of IFN-γ expression in T cells, we first collected pictures of DC–T cell single conjugates with transmitted light/γ-tubulin staining channel (red or blue depending on the experiment), and then we measured the position of the MTOC spot to score it as polarized or not. We next switched on the green channel to visualize the signal of IFN- γ in the T cell.

ELISA

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

Cytoskeletal disruption

To inhibit microtubule polymerization, cells were treated with 1 µg/ml colchicine (Sigma-Aldrich) 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.

Silencing of Dlg1, Cdc42 and expression of Cdc42 mutants

The control siRNA (ATTCTATCACTAGCGTGAC) and specific Cdc42 siRNA (GGGCAAGAGGATTATGACATT) were as previously reported (Malacombe et al., 2006; Momboisse et al., 2009). For Dlg1 depletion we used a commercial SMARTpool siRNA (Dharmacon, USA). $6x10^6$ DCs were transfected with 1 µM of siRNA using the Amaxa Nucleofector according to the manufacturer's instructions. Cells were collected 48 or 72 h after transfection. For the expression of

GFP-tagged construct of shCdc42, Cdc42 WT or N17 (Gasman et al., 2004), $1x10^7$ DCs were transfected with 2–3 µg of endotoxin-free DNA. Cells were collected after 48 h and live GFP+ cells were enriched by cell sorting. For rescue experiments, DCs were cotransfected with siRNA directed against Cdc42 and a plasmid coding for the HA-tagged mutated rescue Cdc42, resistant to siRNA degradation, which has been generated by mutagenesis (QuickChange mutagenesis kit; Stratagene) of the codon GAT encoding Asp-63 to GAC. To assess depletion of Cdc42 or Dlg1, $1x10^5$ cells were lysed and analyzed by SDS page using anti-Cdc42 antibody (BD) or rabbit-anti Dlg1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To assess maturation and cytokine production, $1x10^5$ cells were plated for 4 h in TLR agonist–containing medium. The levels of IL-12 were measured in the cell culture supernatants by ELISA. The up-regulation of surface maturation markers (MHC class II, B72 and CD40) was evaluated by FACS analysis at 4 and 12 h after transfection.

STAT4 phosphorylation

For analysis of STAT4 phosphorylation by Western blot, $2x10^5$ control of Cdc42-silenced DCs were mixed with $4x10^6$ OT-I/CD45.1 in 96-well plates by spinning at 800 rpm for 1 min. After 30 min of incubation at 37°C, the cells were lysed and cell lysates were resolved by 10% SDS-PAGE. Membranes were blocked in TBS-5% BSA and developed using anti pSTAT4-ser721 (sc-22160; Santa Cruz Biotechnology, Inc.), followed by anti–rabbit horse radish peroxidase (Sigma-Aldrich). For FACS analysis, DC–T cell synapses were formed as for STAT6 analysis. At the end of incubation at 37°C, cells were fixed with 1% PFA (10 min) and permeabilized with methanol 80% (20 min). T cells were incubated with supernatant of TLR-stimulated DCs as a control (referred to as soluble IL-12 in Fig. 5 B). After washing, cells were stained with Alexa Fluor 488 mouse anti-STAT4 (pY693; BD) or mouse IgG-FITC isotype control (BD), CD45.1-rhodamine (1:400), and CD8-Cy5 (1:400). For FACS analysis, we gated on CD8- CD45.1 double-positive events. Inside this population, T cells alone or conjugated with DCs were distinguished by the FSC/SSC profile.

IFN-γ mRNA production

For analysis of IFN-γ mRNA production by qRT-PCR, $2x10^5$ Control of Dlg1-silenced DCs were stimulated with CpG+LPS (10µg/ml) for 4h and loaded with 10nM MHC-class I restricted OVA peptide (SINFEKL). The cells were then washed and mixed with $4x10^5$ OT-I cells in 96-well plates by spinning at 800 rpm for 1 min. After 1h of incubation at 37°C, the cells were lysed and RNA was isolated by

standard protocols. DCs alone (immature and mature) and T cells alone (loaded or not loaded with peptide) were used as the controls.

FACS analysis of IFN-γ in synapses

Control of Cdc42-silenced DCs were stimulated with 10 µg/ml CpG+LPS and loaded with 10 nM OVA class I peptide. As controls, we used DCs without TLR agonist or without peptide. After 3 h of stimulation, DCs were washed extensively to eliminate extracellular IL-12 and mixed with OVA-specific OT-I cells. After 30 min or 2 h of interaction, cells were fixed, and the cell surface was labeled with anti CD8 antibodies. Cells were permeabilized and labeled with Alexa Fluor 488 anti–mouse IFN-γ (XMG1.2; eBioscience) or the corresponding isotype control. For FACS analysis, we gated on CD8+ events, and inside this population, T cells alone or conjugated with DCs were distinguished by the FSC/ SSC profile. Values are expressed as the percentage of cells positive for IFN-γ in respect to the corresponding isotype control.

In vitro migration assay

DCs migration capacity was evaluated using 24-well Costar Transwell chamber (5 µm pore size) (Corning). Control or Dlg1-depleted DCs ($2x10^5$), stimulated with LPS (10 µg/ ml) for 3h were resuspendend in 100ul of medium and placed in the upper insert. 500ul of medium containing 100ng/ml of CCL19 or CCL21 was added to the lower well. The chambers were incubated at 37° C and 5% CO_2 for 3h. The cells that migrated were recovered from the lower well, counted and the number is expressed as the percent of input cells.

In vivo migration assay

Control or Dlg1-depleted DCs were harvested 48h after transfection and labeled with 4µM of 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE), according to manufacturer's instructions. $3x10^5$ of DCs were then injected into the footpad of C57B/6 mice. 24h later the cell suspension from the draining lymph node was obtained by a treatment with collagenase D. The cells were then stained for CD11c expression and the percent (%) of CD11c+/CFSE+ cells over total CD11c+ DCs present in the lymph node was assessed by FACS.

T cell activation and cytokine production

To analyze T cell proliferation, IL-2 and IFN-γ production, $1.2x10^4$ of Control, Dlg1-depleted DCs, Cdc42-depleted, DCs expressing WT or dominant-negative N17 mutant or DCs cotransfected with silencing and rescue plasmid were plated on 96 wells and stimulated for 4 h with 1 µg/ml CpG/LPS. Cells were loaded with serial dilution of SIINFEKL peptide. After 4 h, cells were washed and $1x10^5$ OT-I/CD45 that had been prelabeled with CFSE were added to the culture wells. For early activation experiments, supernatants were collected at day 1 and cells were labeled with anti-CD8, anti-CD45.1 and anti-CD69 antibodies. For later T cell events, the supernatant was collected at day 3 and the cells were labeled with anti-CD8 and anti-CD45.1 antibodies to gate on T cells. All cells were acquired to determine the CFSE dilution profile, and the total number of cells in each well. To determine the intracellular levels of IFN-γ at day 3, cultures were restimulated with 10 µg/ml BFA and 1µM OVA class I peptide for 4 h. Cells were fixed and the surface was labeled with anti-CD45.1/ anti-CD8 antibodies, permeabilized, and labeled with Alexa Fluor 488 anti–mouse IFN-γ (XMG1.2; eBioscience). The content of IL-2 (day 1) or IFN-γ (day 3) in cell culture supernatants was analyzed by ELISA using standard procedures.

Adoptive transfer and T cell activation

$2x10^6$ of OT-1/CD45 T cells or OT-1 labeled with 4µM CFSE were injected *i.v.* into the recipient mice. $3x10^5$ of Control or Dlg1-depleted DCs were pulsed with 0.5nM MHC class-I-restricted OVA peptide (SINFEKL) for 3h in complete medium at 37°C 5%CO2, washed and injected into the footpad 24h upon T cells transfer. For the early activation, draining lymph nodes were collected after 1 day, digested in collagenase and cell suspension was labeled with anti-CD8, anti-CD45.1 and anti-CD69 antibody. In order to assess T cell priming, draining lymph nodes were collected 3 days later, digested in collagenase and i) the percentage of transferred (OT-1/CFSE+) over endogenous (CD8+) population ii) and CFSE dilution profile of transferred OT-1 cells was assessed by FACS.

Intracellular IFN-γ staining

Intracellular IFN-γ production by primed CD8+T cells was evaluated using lymph nodes cells, cultured for 4h with 2µM of SINFEKL peptide or medium alone in the presence of brefeldin A (7µM/ml) (BD Biosciences). Cells were stained with anti-CD8 antibody and anti-CD45.1 antibody for 25min at 4°C.

After fixation with PermFix (BD Biosciences) cells were stained for intracellular IFN-γ in PermWash solution (BD Biosciences) for 40min at 4°C.

Statistical analysis

All data were reported as the mean ± SD as calculated using GraphPad Prism 5 software. The unpaired Student's *t* test was used as indicated in the text to assess significance.

Results

Aims of the thesis

Dendritic cells (DCs) are antigen-presenting cells (APC) essential to ensure adequate awakening of the immune response as well as the induction of tolerance. DCs integrate pathogens information and undergo an intrinsic program of maturation which renders them capable to present pathogen-derived sequences to naïve T cells within the immunological synapse (IS). Furthermore, the maturation program endows DCs with a high level of morphological and functional plasticity which have been shown essential for proper T cell fate determination. Within immunological synapse, DCs polarize their cytoskeleton, upregulate and cluster signaling and adhesion molecules and temporally regulate activation of signaling cascades; thus ensuring the specificity and amplification of the signals during contact with T cells. Recent *in vivo* studies revealed that another important aspect of DCs maturation program is their ability to mediate contact duration with T cells; a feature shown to be directly responsible for the induction of immune response or tolerance.

Three types of signals are transmitted to T cells at the IS: MHC peptide complexes (signal 1), costimulatory molecules (signal 2) and cytokines (signal 3). How DCs can coordinate in time and space the delivery of these three signals has been little explored.

In this thesis we investigated whether establishment of cell polarity and polarized secretion may operate in DCs as a mechanism to optimize signal transmission at the IS. We studied polarization of the microtubules cytoskeleton in DCs to decipher the molecular network that ensures establishment of polarity and to determine the relevance of DCs polarization for T cell priming. We focused on polarization at the IS and synaptic delivery of IL-12, a key cytokine ensuring fate determination in T cells, whose mechanisms of secretion are still unknown.

3.1. The functional aspect of MTOC polarization in DCs during synapse formation

To gain further insight into DC properties as antigen presenting cells, we asked whether the microtubule system of DCs becomes polarized during the interaction with T cells, and what would be the outcome of such a process. In order to answer to these questions we have established and *in vitro* model system of the immunological synapse using murine DCs differentiated from bone-marrow and antigen specific naïve T cells isolated from TCR transgenic mice. MTOC polarization was analyzed using confocal imaging and functional assays to study the consequences of MTOC polarization on T cell. Here I will briefly summarize published findings from our lab.

3.1.1. DCs polarize MTOC/IL-12 toward antigen-specific synapse with T cells

Initially, we examined the reorganization of the microtubule cytoskeleton in DCs during interaction with naive OT-1 cells. Bone marrow–derived DCs were activated by TLR agonists to induce maturation and were loaded or not loaded with OVA class I peptide. DCs were plated on a fibronectin matrix and OT-I cells were layered over DCs for 30 min. After washing off nonadherent cells, slides were fixed and analyzed by confocal microscopy. To visualize the microtubule cytoskeleton, we performed immunolabeling with anti-tubulin antibodies. DCs differentiated from the bone marrow of centrin GFP knock-in mice were used to allow a sharper visualization of the MTOC. In several DC–T cell conjugates, we observed the DC's MTOC ("*DC-MTOC"* hereafter) in close proximity to the synaptic membrane. The percentage of cells showing a polarized MTOC was quantified according to the criteria described in the *Material and methods*. As shown in Fig.1a and b, DC-MTOC polarization depended on antigen dose. Only a 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. Thus, DCs engaged in antigen-specific synapses undergo remodeling of the microtubule cytoskeleton by redirecting the MTOC toward the interacting T cell in an antigen dose-dependent manner.

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 the formation of stronger and longer lasting DC–T cell interactions that in turn depend on an intact actin cytoskeleton (Benvenuti et al., 2004a; Benvenuti et al., 2004b). To understand whether microtubules were preferentially polarized in mature DC–T cell contacts, we formed synapses using antigen-loaded DCs (1 nM peptide) that were incubated or not with TLR agonist before mixing with antigen specific T cells.

DC–T cell conjugates formed by DCs that had not been stimulated by TLRs agonist showed a low degree of MTOC polarization. As soon as 2 h after activation, the number of conjugates with the MTOC facing the T cell increased, reaching maximal levels at 6 h after stimulation (Fig1c).

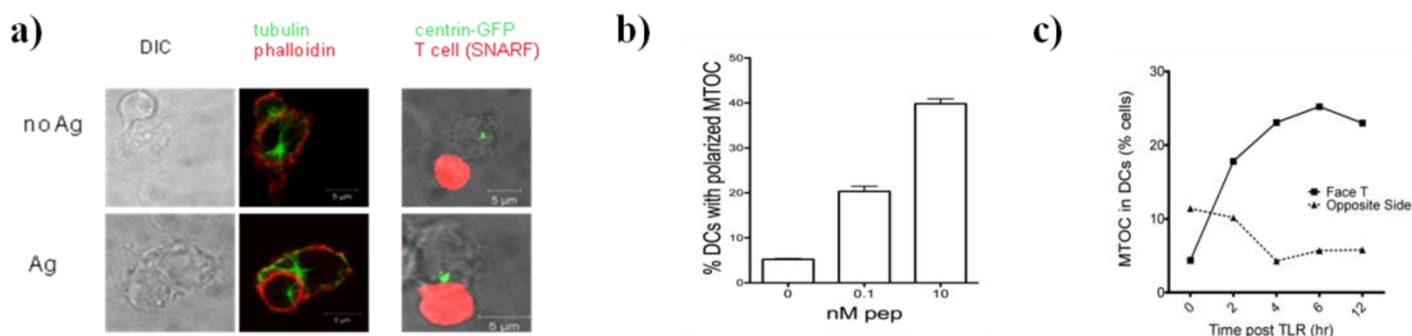

Figure 1. MTOC polarization in DCs during synapse formation a) DCs were stimulated with a combination of TLR agonists (CpG and LPS) and loaded (Ag) or not (no Ag) with OVA class I peptide before mixing to OVA-specific CD8+ T cells (OT-I). Confocal sections showing the MTOC position detected by staining with anti–α-tubulin antibodies (green, left) or using DCs differentiated from centrin-GFP knock-in mice (green, right) mixed with carboxylic acid, succinimidyl ester (SNARF)–labeled T cells (red). **b)** DC–T cell conjugates were formed using TLR-stimulated DCs loaded with the indicated doses of peptide. The percentage of DCs with the MTOC polarized toward the DC–T cell interface was quantified as described in the experimental procedure. Values are plotted as means ± SEM of >100 conjugates/condition. **c)** DCs were treated with TLR agonist for the indicated periods and loaded with 1 nM peptide before synapse formation. The percentage of polarized DCs was scored in at least 50 conjugates for each condition in 3 independent experiments.

MTOC polarization has been functionally associated to directed secretion of cytokines and lytic granules in T cells and NK cells (Chen et al., 2006; Stinchcombe et al., 2006) We thus asked whether DC-MTOC polarization is functionally linked to polarized secretion at the synapse. To test this hypothesis we focused on IL-12, a cytokine that is produced in high amounts by DCs upon TLR stimulation. IL-12 has key roles in Th1/2 fate determination of CD4+ T cells and it is involved in clonal expansion and survival of CD8+ T cells (Pearce and Shen, 2007; Trinchieri, 2003; Valenzuela et al., 2002). We first analyzed the kinetics of production and the intracellular distribution of IL-12 in DCs after TLR engagement. The bioactive form of IL-12 is the heterodimeric IL-12p70 composed of the p40 and the p35 chains. Cells were pulsed with TLR agonist and harvested at different time points to analyze IL-12p40 and IL-12p70 content in supernatants and cell lysates. As shown in Fig. 2a, both

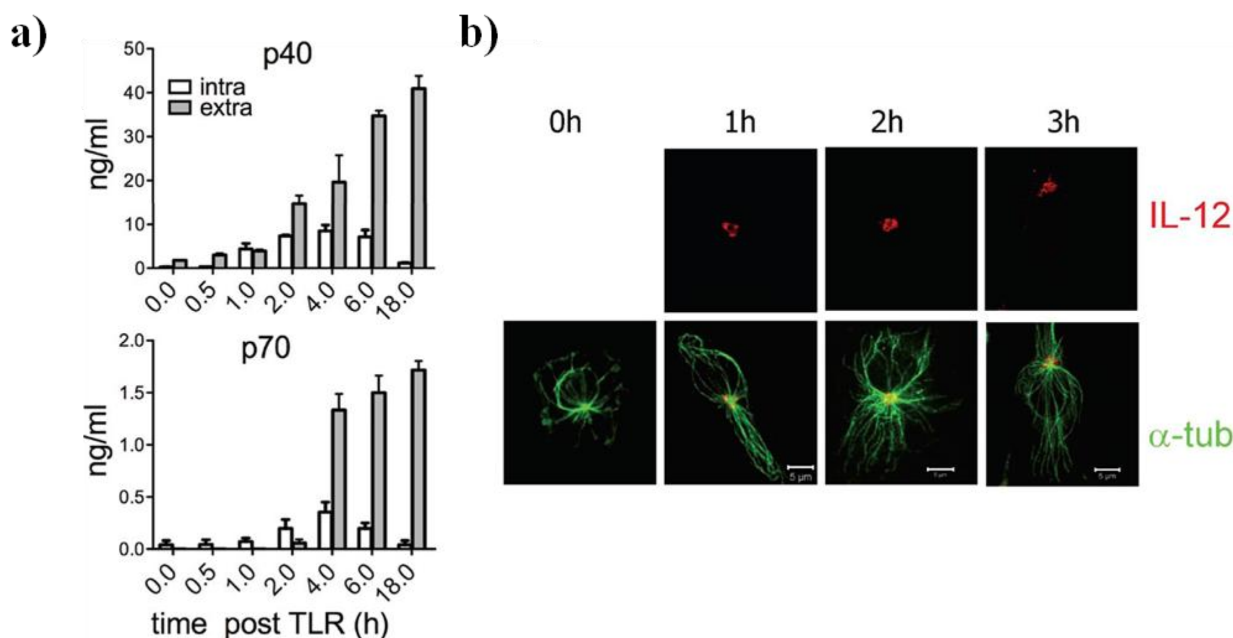

Figure 2. IL-12 associates with MTOC at different (all) time points post TLR-stimulation. A) Kinetic of IL-12 production and secretion in DCs. DCs were stimulated with a combination of TLR agonist for the indicated times (time post TLR). The relative content of IL-12 p40 and p70 in cell culture supernatants (gray bars) and cell lysates (white bars) was determined by ELISA. Bars show means ± SEM of five independent experiments. **b)** Intracellular localization of IL-12 in DCs. DCs stimulated with TLR agonist for the indicated times were immunolabeled with anti–α-tubulin (green) and anti–IL-12 p40/70 (red) antibodies. Images are z projections of confocal sections.

IL-12 p40 and IL-12p70 are present in the intracellular fraction as early as 1 h after stimulation and reach the highest intracellular concentration at 4–6 h after TLR ligation. At 18 h, the intracellular content has been almost completely emptied. In agreement, intracellular staining by FACS showed a peak in the number of IL-12–positive cells at early time points (4 h after TLR = 45 ± 3%) that declined with time (35.4 ± 4% at 6 h and 7.57 ± 5% at 18 h; not depicted). Confocal analysis of the intracellular distribution of IL-12 (revealed using an anti p40/p70 antibody hereafter referred to as "*anti–IL-12*") showed that most of the IL-12 signal is found in a ring that surrounds the MTOC at 1, 2, and 4 h after TLR induction with little punctuate staining in other areas of the cell (Fig. 2b). Staining with anti-giantin antibodies indicates that IL-12 signal localizes with the Golgi complex around the MTOC (not depicted). Collectively, these data show that a few hours after TLR ligation intracellular IL-12 is mostly distributed around the MTOC in DCs.

We next asked whether the intracellular pool of IL-12 is translocated to the synaptic region in concert with MTOC polarization when DCs encounters T cells. DCs were stimulated with TLR agonists for 5 h to reach the highest intracellular levels of IL-12. DCs were loaded with OVA peptide and mixed with OVA-specific CD8+ T cells (prelabeled with CFSE to facilitate detection of DC–T cell

couples) for 30 min to allow synapse formation. In DC–T cell antigen-specific conjugates, intracellular IL-12 remained tightly associated to the MTOC and it was transported to the synaptic region close to the T cell membrane (Fig. 3a). In the presence of peptide, up to 67% of conjugates showed enrichment of IL-12 at the DC–T cell interface. We also found a proportion of cells (21 ± 3%; not shown) with polarized IL-12 and a not fully oriented MTOC, suggesting that IL-12 vesicles may also travel in front of the MTOC. Pretreatment of DCs with colchicine significantly inhibited recruitment of IL-12 at the IS (Fig. 3b), indicating that MTOC and IL-12 polarization are linked.

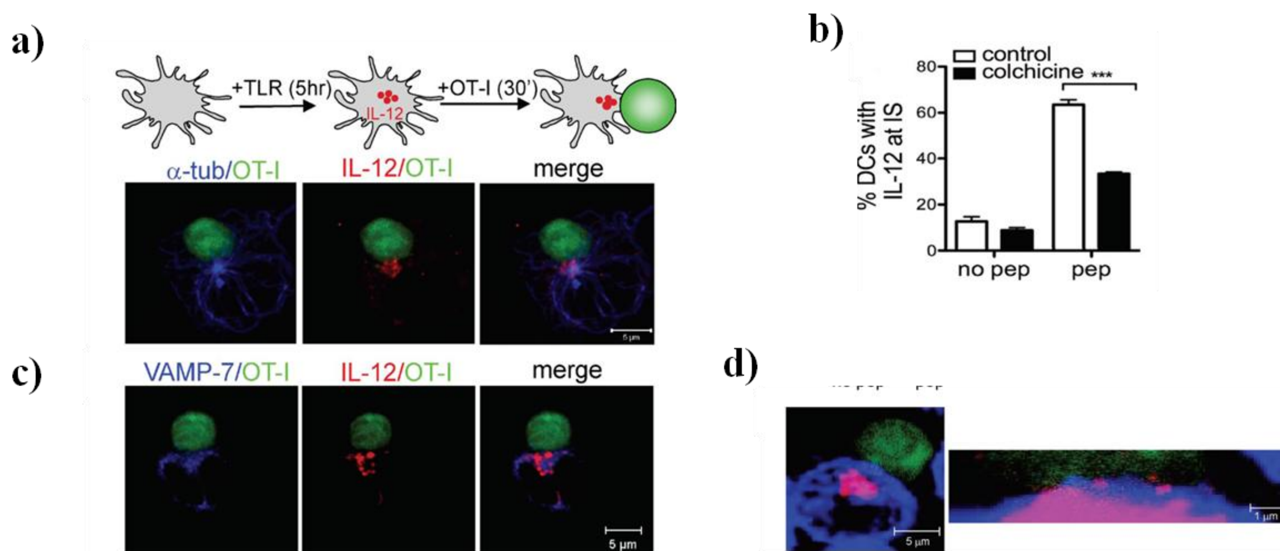

Figure 3. Polarization of IL-12 at the synapse. a) Confocal images showing recruitment of IL-12 at the DC–T cell interface. Experimental outline: DCs were stimulated with TLR agonist (5 h), loaded with peptide, and mixed with OT-I cells to induce synapse formation. (top) Confocal planes showing IL-12 (red) and α-tubulin (blue) distribution in DC–T cell doublets. T cells are in green (CFSE labeling). **b)** DCs were treated with colchicine before synapse formation. The percentage of conjugates with IL-12 enriched at the contact in control and treated cells was measured in at least 50 conjugates/condition in three independent experiments ($P < 0.001$, Student's *t* test). **c)** Labeling with anti–VAMP-7 (Ti VAMP) and anti–IL-12 antibodies on fixed DC–T cell synapses shows alignment of secretory organelles close to the DC–T cell interface. **d)** DCs prelabeled with WGA (blue) to demark the membrane were mixed with CFSE-labeled T cells. The image shows one representative high-magnification projection of z stacks along the contact zone which depicts vesicles of IL-12 (red) crossing the DC plasma membrane.

In NK and T cells, it has been shown that different trafficking routes are associated with polarized or multidirectional delivery of soluble mediators toward a target. However, whereas cytotoxic granules are released in a strictly polarized fashion in NK and cytotoxic T cells, the release of cytokines seem to be less spatially confined. For instance, in NK cells interacting with a target, IFN-γ is released toward the synapse, but it can also be released elsewhere. Two evidences indicate that in DCs recruitment of the

intracellular pool of IL-12 toward the IS leads toward preferential secretion in the synaptic area (not necessarily exclusive). First, we observed that VAMP-7–positive vesicles (VAMP-7 is a Ti-VAMP that marks late endocytic vesicles and mediates fusion of intracellular vesicles with the plasma membrane (Braun et al., 2004), were enriched at the DC–T cell interfaces of antigen specific conjugates in close proximity to IL-12–positive vesicles, suggesting that secretory organelles align at the contact site (Fig. 3c). Second, high magnification sections taken through the interaction site revealed vesicles of IL-12 crossing the DC membrane toward the T cell (Fig. 3d). Thus, DC polarization reorients the secretory apparatus and increases the concentration of IL-12 vesicles directed toward the IS. Therefore, we conclude that at early time points after infection, intracellular IL-12 is dragged at the DC–T cell contact site during antigen recognition in a microtubule-dependent fashion. These data suggest that polarization of cytokines-containing vesicles may have functional significance and induce citokine dependent signaling in T cells.

3.1.2. IL-12 signaling at the synapse

During my thesis I focused my effort in defining functional significance of MTOC polarization in DCs. The first assay was developed to test very early events of IL-12-dependet signaling induced in T cells by polarized IL-12. Binding of IL-12 to its receptor initiates a signaling cascade that, via the Janus-associated kinases, leads to phosphorilation of the STAT4 transcription factor and transactivation of IL-12 regulated genes, such as IFN-γ (Bacon et al., 1995a). Moreover, downstream target of IL-12 include activation of Bcl-2 and Bcl-3, which promote the survival of antigen-activated CD8+ T cells, inducing their clonal expansion (Li et al., 2006). To investigate the functional impact of IL-12 recruitment at the IS, we studied IL-12–dependent events in T cells. To this aim, we set up an assay to measure phosphorylated STAT4 (pSTAT4) in T cells during synapse formation. DCs were left untreated (immature) or stimulated with TLR agonist for 5 h (mature) and loaded or not with peptide. DCs were then mixed with antigen-specific OT-I cells and lysed after 30 min 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 that do not contain IL-12. Most importantly, T cells mixed with mature DCs pulsed with antigen showed higher pSTAT4 levels than T cells incubated in the absence of antigen (Fig. 4a), indicating that synapse formation promotes STAT4 activation. To quantify the extent of pSTAT4 signaling, we used intracellular staining and FACS analysis. DCs were pulsed or not pulsed with TLR agonist and peptide and mixed with T cells for 30 min. The percentage of pSTAT4-positive T

cells was determined by gating on the region of DC–T cell doublets and, as a control, on T cells not engaged in synapse, as described in the experimental procedure. T cells alone showed a low background level similar in all cases. In the gate of DC–T cell doublets, we observed an overall higher background and a specific pSTAT4 signal that varied depending on the DC state. Immature DCs induced an equivalent signal regardless of the presence of peptide. In contrast, incubation with mature DCs induced a clear increase in the number of pSTAT4+ T cells that was significantly higher in conjugates formed with antigen loaded DCs as compared with not loaded DCs (P = 0.0029; (Fig. 4b). Treatment with colchicine to disrupt IL-12/MTOC association and translocation to the IS caused a significant reduction in the number of pSTAT4+ T cells in antigen-specific conjugates (Fig. 4c)

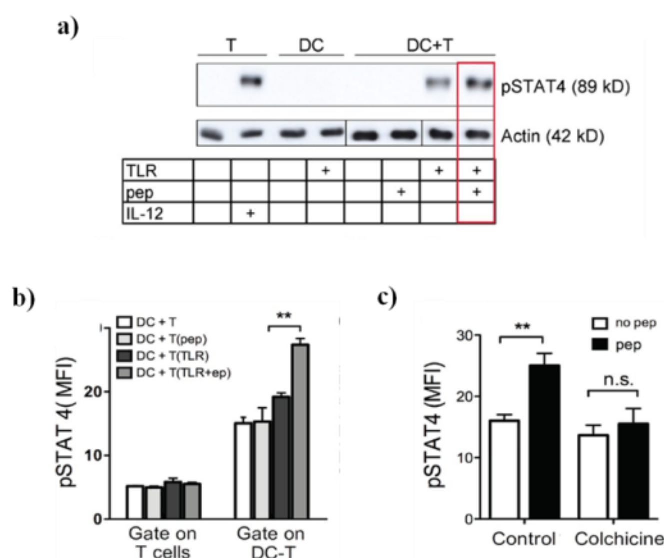

Figure 4. Antigen-specific synapse formation induces STAT4 signaling in T cells. a) DCs were pretreated with TLR agonist, loaded with 10 nM of OVA peptide, and mixed with OT-I cells. After 30 min of incubation, cells were lysed and analyzed by Western blotting using an antibody against pSTAT4. Control 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 are DCs coincubated with T cells in different conditions as indicated in the table. **b)** Detection of pSTAT4 by intracellular FACS analysis. DCs were either left untreated or stimulated with TLR agonist (TLR), loaded (pep) or not with OVA peptide, and mixed with OT-I for 30 min. Cells were fixed and labeled intracellularly with anti-pSTAT4 Alexa Fluor 488 antibody. The MFI was determined by gating on isolated T cells (gate on T cells) or on DC–T cell doublets (gate on DC–T cell) as described in experimental procedure. Data represent the mean values ± SEM (subtracted for the isotype control values) obtained in three independent experiments. **c)** Control DCs or DCs treated with colchicine were used in the assay described in B. Data show the MFI ± SEM of pSTAT4 signal determined on T cells engaged in doublets in four independent experiments ($P < 0.01$; Student's *t* test).

To causally link polarization of the intracellular pool of IL-12 to activation of IL-12–dependent events, we performed analysis at the single cell level. As the signal of pSTAT4 was too weak to allow quantification by immunofluorescence, we tracked neosynthesis of IFN-γ, the first gene induced by IL-12 in a STAT4-dependent manner (Lund et al., 2004). DCs were stimulated to induce

accumulation of intracellular IL-12, washed extensively, and mixed with OT-I cells for either 30 min or 2 h. At 2 h, a clear IFN-γ+ ring focused around the T-MTOC was visible in many T cells engaged in synapses (Fig. 5a). IFN-γ in DC–T cell conjugates was specifically induced by 2 h of interaction with IL-12 containing DCs, as no signal was detected when T cells were incubated with immature DCs, when the interaction was stopped after 30 min, or when T cells and DCs were mixed in the absence of antigen (Fig. 5 b). Importantly, single-cell quantification by confocal microscopy showed that IFN-γ was preferentially induced in T cells in synapse with polarized DCs (Fig. 5 c). Therefore, synapse formation and polarization of the intracellular pool of IL-12 controls activation of STAT4 and IFN-γ neosynthesis in T cells.

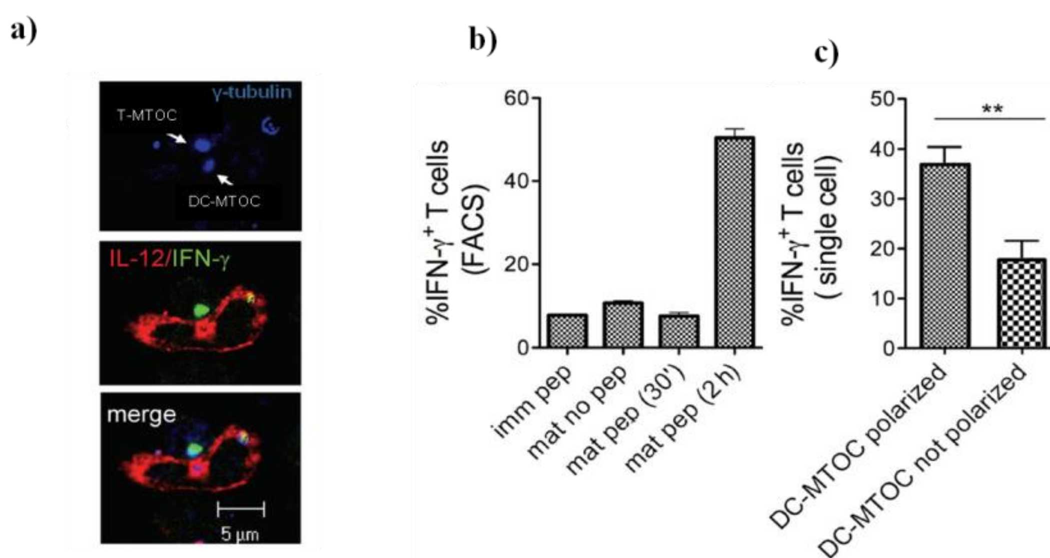

Figure 5. Antigen-specific synapse formation induces IFN-γ neosynthesis in T cells a) DC–T cell antigen- specific interactions trigger IFN-γ neosynthesis in T cells. DCs activated by TLR agonist and loaded with peptide were allowed to interact with OT-I for 2 h, fixed, and labeled. Confocal images show polarized IL-12 (red) and DC-MTOC (blue) facing IFN-γ staining around the T cell MTOC (green). **b)** Quantification of IFN-γ neosynthesis in T cells engaged in antigen-specific synapses was measured by intracellular FACS analysis under different conditions (as specified). Values plotted indicate the percentage of IFN-γ+/CD8+ T cells gated in the region of DC–T cell doublets (isolated T cells show no IFN-γ signal), subtracted for the isotype control (one of three experiments with identical results is presented). **c)** Early expression of IFN-γ in T cells correlates to DC-MTOC polarization at the IS. Cells were labeled as in **a)** and quantified. Bars show the distribution of IFN-γ–positive T cells in synapse with DCs that present (polarized) or not (not polarized) the MTOC facing the T cell membrane. The data comes from quantification of >100 cells in three independent experiments (**, P = 0.052).

3.1.3. WASp as a putative model to follow MTOC polarization

To fully evaluate functional significance of MTOC polarization, we next looked for the ways to block it and analyze the effect on T cell activation. Wiskott-Aldrich protein (WASp) induces actin polimerization through the activation of Arp2/3 nucleation complex (Orange et al., 2004). WASp activity has been shown crucial in various context of proper functioning of hematopoietic cells *e.g.*

cellular migration (Castellano et al., 1999; de Noronha et al., 2005), lymphocyte morphology (Kenney et al., 1986), and synapse formation both in T cells (Zhang et al., 1999) and in DCs (Bouma et al., 2011). Recent advances regarding polarity in hematopoietic cells indicate WASp as one of the factors contributing to microtubular cytoskeleton movement during synapse formation (Banerjee et al., 2007).

To assess whether WASp is implicated in the MTOC movement toward the synapse in DCs, we evaluated MTOC location in bone marrow derived DCs obtained from WASp knockout mice. DCs were stimulated for 5h with TLR agonists in the presence of increasing doses of antigen. At the end of the stimulation period OT-1 cells were added for 30min. The synapses were then fixed and stained with the antibody recognizing α-tubulin. The occurrence of MTOC polarization in DCs toward the synapse with T cell was then quantified applying the criteria described in *Materials and Methods*. In line with previous observations (Fig 1), wtDCs show an increase in MTOC polarization toward the synapse that correlated with the increase in the antigen dose (from 5% in the absence of antigen rising to 40% in the presence of 10nM peptide). On the other side, however, WASp-/- DCs experienced significantly lower incidence of MTOC polarization (from 3% in the absence of peptide to 14% in the presence of 10nM peptide), corresponding to a reduction of 65% (Fig6).

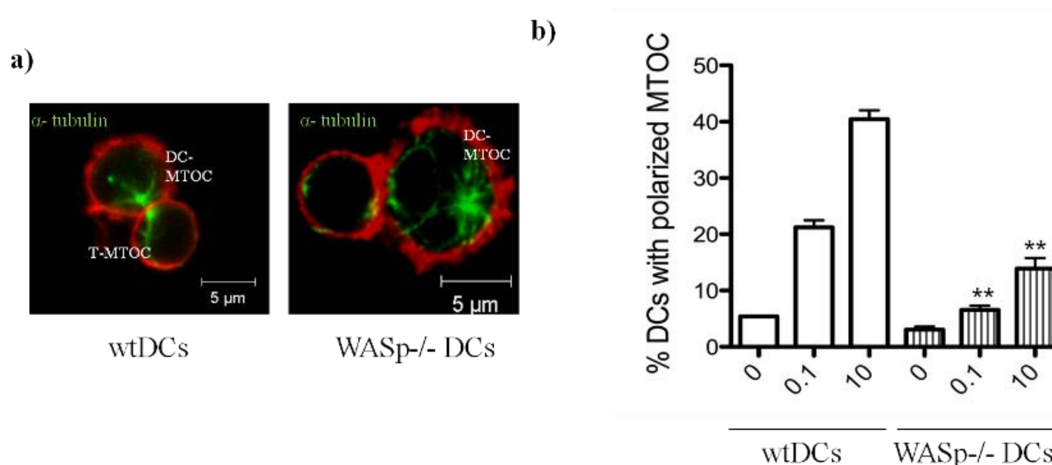

Figure 6. WASp-/- DCs polarize MTOC less readily then wtDCs. a) Representative images demonstrating the position of MTOC in wtDCs and WASp-/- DCs, relative to the synapse with T cells. DCs were stimulated with the mix of TLR agonists (CpG and LPS) for 5h in the presence of 10nM peptide. DCs were then mixed with OT-1 cells for 30min. The image represents confocal sections showing the MTOC position detected by staining with anti–α-tubulin antibodies **b)** DC–T cell conjugates were formed using TLR-stimulated DCs (wtDCs and WASp-/-DCs) loaded with the indicated doses of peptide. The percentage of DCs with the MTOC polarized toward the DC–T cell interface was quantified as described in the experimental procedure. Values are plotted as means ± SEM of >100 conjugates/condition

Given that WASp-/- DCs are impaired in MTOC polarization toward the synapse with T cells, we reasoned that it would be a good model to test the functional consequences that the absence of MTOC translocation could have on T cell side. In order to exclude other intrinsic dysfunctions due to the absence of protein (WASp), we measured the levels of IL-12p40 and IL-12p70 after 3h and 6h of stimulation. At the end of the stimulation period, cytokine content was measured both in the supernatants as well as intracellularly. The intracellular levels of IL-12p40 peaked at 3h post stimulation and decreased at 6h, whereas the protein levels increased in the supernatant. The levels and secretory rate of IL-12p40 were only slightly decreased in WASp-/- DCs. In contrast, we observed a strong decrease in the levels of IL-12p70, both intra and extracellularly in WASp-/-DCs (Fig7a). That suggested that WASp-/- DCs had impairment either in cytokine secretion and/or its production. In order to address that question we measured the levels of IL-12p40 and IL-12p70 transcripts by qRT-PCR. We stimulated DCs (wt and WASp-/-) with TLR agonists for 2h, 4h and 6h. As shown in Fig7b, IL-12p40 mRNA levels were slightly reduced in WASp-/-DCs, whereas we observed a strong reduction in IL-12p70 mRNA levels 4h and 6h post stimulation. Together these data indicate that production of the IL-12p40 subunit and especially of the limiting IL-12p35 subunit is inhibited in WASp-/-DCs at the level of gene induction which is reflected in lower concentration in the culture supernatants.

In conclusion, the results obtained from WASp-/- DCs did show that WASp deficiency affects MTOC translocation in DCs. However, these cells also had further problems in general production of IL-12, hampering its use as a model to evaluate the consequence of IL-12 polarization on T cell activation. Nevertheless, these initial observations led to a different project, aimed to elucidate whether this perturbation of cytokine production in WASp-/- DCs might have an impact on the immune response in WASp-/- mice.

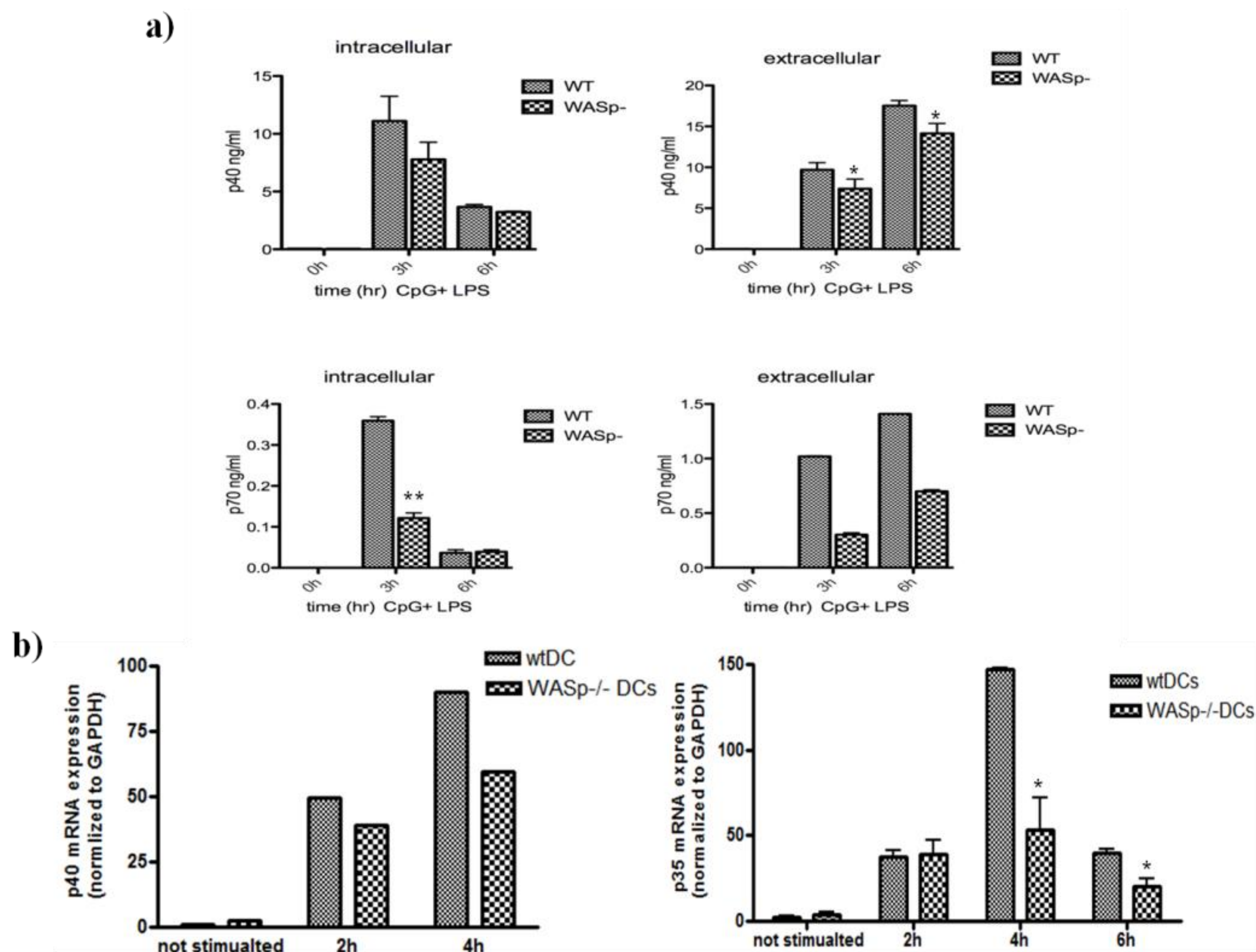

Figure 7. WASp-/- DCs have a defect in IL-12 production. a) DCs (wt and WASp-/-) were stimulated with the mix of TLR agonists (LPS and CpG) for the indicated periods of time. After that the supernatants were taken and the cells were lysed. The concentration of cytokines IL-12p40 and IL-12p70 in these two fractions was measured by ELISA. Graphs represent intra- and extracellular levels of IL-12p40 (*upper panel*) and IL-12p70 (*lower panel*) in wtDCs and WASp-/- DCs. **b)** DCs (wt and WASp-/-) were stimulated as in **a**). After that, the cells were lysed and the levels of cytokines production were measured by qRT-PCR. A representative graph of 2 independent experiments is shown for IL-12p40. The values on the IL-12p70 graph are represented as means ± SEM of 2 independent experiments (*, p=0.0310).

3.1.4. Cdc42 regulates MTOC polarization toward the synapse

As a second model to inhibit MTOC translocation in DCs, we focused on Cdc42. Cdc42 is a small Rho GTPase, crucially involved in regulating cell polarity in various cells (Etienne-Manneville, 2004). Previous studies on the cells of the immune system showed that Cdc42 regulates MTOC polarization in these cells (T cells, NK cells, and macrophages) through multiple effectors that control actin dynamics and anchoring of MT to the plasma membrane, providing the forces that pull the

MTOC (Banerjee et al., 2007; Eng et al., 2007; Stinchcombe et al., 2006). Thus, we went on to check whether the depletion of Cdc42 might have a role in the process of MTOC translocation in DCs.

Initially, to deplete Cdc42 we used a plasmid encoding shRNA against Cdc42 that was fluorescently tagged. As the means of transfection we used the nucleotransfection (Amaxa), as described in *Materials and Methods*. However, even though it had a good side of being fluorescent, Cdc42-GFP delivered as plasmid DNA was very toxic for the cells and had low percent of transfection (Fig8).

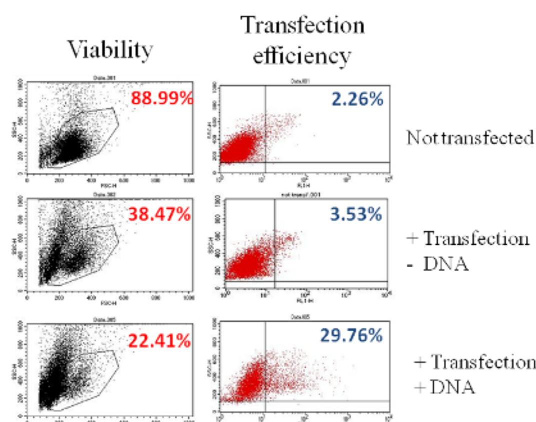

Figure 8. Using DNA encoding shRNA against Cdc42 as a possible tool to impede MTOC translocation. DCs were nucleotransfected (Amaxa) at day 5 of differentiation. 48h later, viability (left column) and efficiency of transfection (right column) were checked by FACS. Mock-transfected DCs were used as the negative control. The viability was checked by gating on DCs according to their size. The transfection efficiency was determined by the presence of fluorescence at FL1 channel.

DNA transfection had a big impact on the cell viability (lowering the number of live cells from 88.99% in not transfected DCs to 22.41% in DNA transfected). Coupled with the relatively low rate of transfection (29.76%), the number of alive and transfected DCs 48h post-transfection was extremely low in order to perform the experiments (6.6% of total cells; not shown).

For the above mentioned motives we next tested transfection of siRNA by Amaxa. As compared to DNA transfection, siRNA was by far less toxic. A Cdc42 specific siRNA previously described (Gasman et al., 2004) efficiently reduced the levels of endogenous Cdc42 in DCs at 48h and 72h after transfection (Fig. 9a). To assess the functional consequences of Cdc42 depletion on cell polarization, DCs were plated on fibronectin and mixed with OT-I cells to induce synapse formation. DCs with reduced levels of Cdc42 showed some morphological differences with a flat and spread shape and, on average, occupied a wider area on the slide. This is in line with a recent report showing that Cdc42-deficient DCs cannot coordinate leading and trailing edge and develop multiple competing leading edges (Lammermann et al., 2009). DC-MTOC polarization was significantly reduced in cells expressing the Cdc42-specific siRNA (51% and 64% of reduction in respect to control cells when cells

were harvested for synapse 48 and 72 h after transfection, respectively) (Fig 9b). Depletion of Cdc42 did not interfere with the association of intracellular IL-12 with the MTOC; however, as expected, translocation of MTOC-associated IL-12 vesicles was reduced in Cdc42- depleted cells (Fig. 9c). Based on these data, we conclude that expression of Cdc42 is required to trigger MTOC and intracellular IL-12 polarization at the DC–T cell interface in antigen-specific conjugates. Another approach we used in order to assess the role of Cdc42 in DCs polarization was to overexpress a plasmid encoding Cdc42 fused to GFP (Cdc42WTGFP) or its corresponding dominant inactive mutant (Cdc42N17GFP). In DCs expressing Cdc42WTGFP, most of the GFP signal was focused at the DC–T cell interface in a pocket-like shape and the MTOC localized together with the peak of GFP intensity. The GFP signal in cells expressing Cdc42N17GFP was more diffused with a central area of high GFP intensity surrounded by bands extending toward the cell periphery (Fig. 9d). The MTOC remained associated to the area of strongest Cdc42 signal, but its translocation was inhibited in respect to cells expressing the WT counterpart (30% of reduction, n = 70 cells analyzed; Fig 9e). Thus, activated Cdc42 is necessary to induce MTOC polarization in DCs.

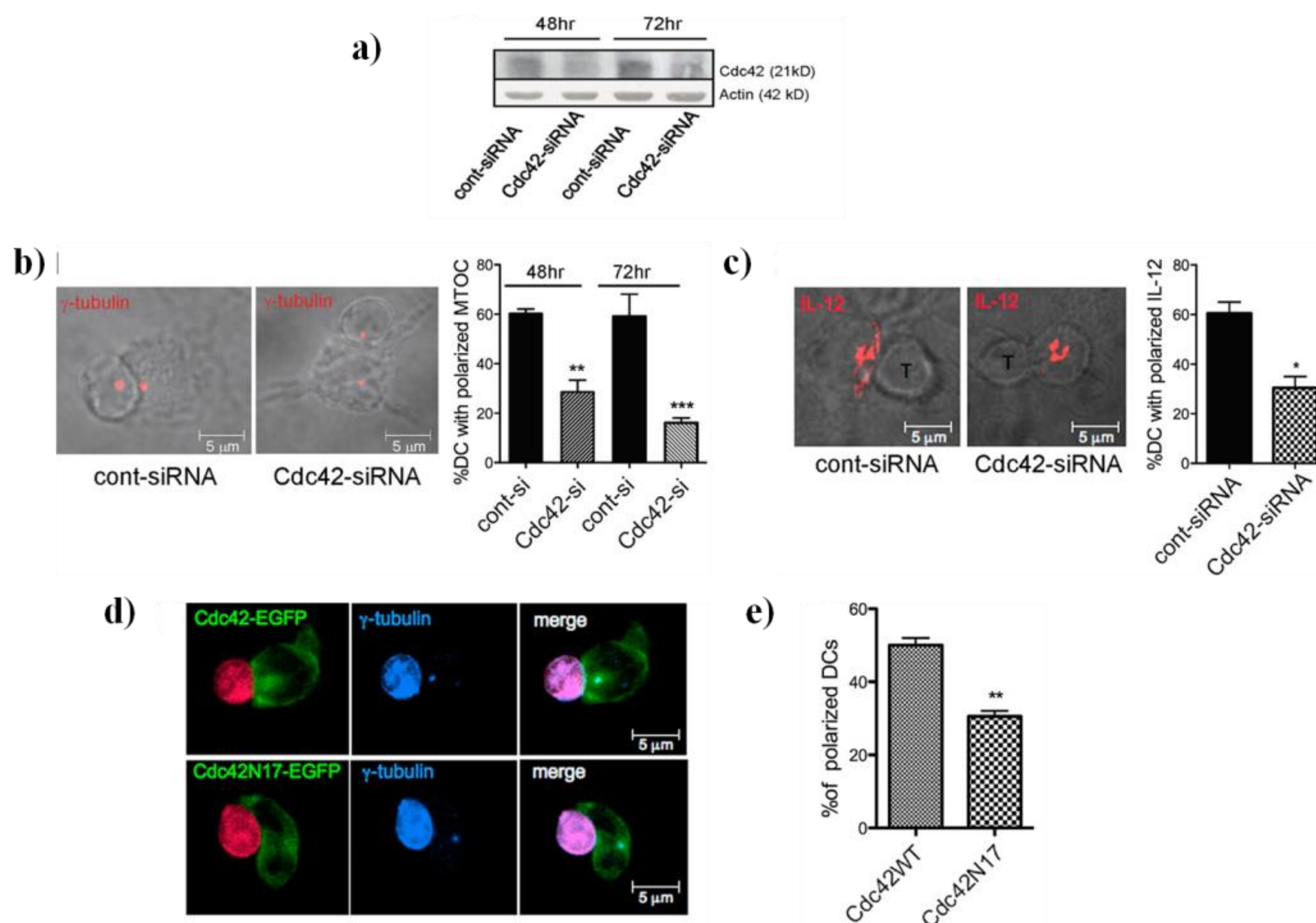

Figure 9. Cdc42 controls MTOC/IL-12 polarization at the IS. Cdc42 is recruited at the IS and is required for DC-MTCO polarization. a) DCs were transfected with a siRNA targeted against Cdc42 (Cdc42-siRNA) or an unrelated siRNA (cont-siRNA). Protein depletion was assessed on total cell extracts by Western blot analysis at 48h and 72 h after transfection. **b)** A representative confocal z slice showing the DC-MTOC position at the IS in control and Cdc42-depleted DCs (anti-γ-tubulin, red). The percentage of DCs showing a polarized MTOC was quantified on at least 90 conjugates in 3 independent experiments. Values are plotted as mean ± SEM (**, P = 0.0032 and **, P = 0.0045 at 48 and 72 h, respectively; Student's *t* test). **c)** Representative images and quantification of IL-12 recruitment at the IS in control and Cdc42-silenced cells. Values are plotted as mean ± SEM of two independent experiments (40 cells/condition; *, P = 0.0365, Student's *t* test) **d)** DCs were transfected with WT (Cdc42WTGFP) or dominant-negative mutant (Cdc42N17GFP) Cdc42 fused to GFP. GFP+ cells were enriched by cell sorting and mixed with SNARF-labeled OT-I cells (emits in the blue and red in this acquisition setting). Confocal images show a representative example of Cdc42WTGFP recruited at the IS and colocalized with polarized γ-tubulin (blue), and one example of diffused Cdc42N17GFP staining in a nonpolarized cell **e)** The percentage of DCs with the MTOC polarized toward the contact region in the two groups was scored in $n > 50$ cells/condition in three independent experiments (**, P = 0.0124).

3.1.5. Assessing the effect of Cdc42-mediate MTOC polarization in DCs on the functionality of T cells

So far, our data suggest that translocation of MTOC/IL-12 to the IS depends on Cdc42. Furthermore, the data indicate that this translocation is linked to T cell activation of IL-12 dependent signals. To firmly establish that Cdc42 dependent MTOC/IL-12 polarization is required for the proper functioning of T cells, we designed functional assays with Cdc42 depleted DC. For this, we used the

assays presented in Fig. 4 to measure early IL-12–dependent signals induced in T cells upon synapse formation. Control and depleted cells were pulsed with TLR agonist, incubated with CD8+ T cells for 30 min, lysed, and probed for the levels of phosphorylated STAT4. Antigen specific induction of pSTAT4 in T cells was observed in control cells but not in T cells incubated with Cdc42-depleted DCs (Fig. 10a). Importantly, the activation of IFN-γ neosynthesis after 2h of interaction was significantly reduced in T cells stimulated by Cdc42-silenced cells as compared with cells treated with control siRNA (Fig. 10b). To rule out that depletion of Cdc42-disturbed DC–T cell interaction, we measured conjugate formation by FACS. As shown in Fig. 10c, DCs and T cells aggregate in a peptide dose-dependent manner. Conjugate formation was not hampered by Cdc42 depletion at the different peptide doses tested; instead, we observed a slight increase in the percentage of T cells engaged in synapses with silenced cells. Hence, we conclude that blocking DC-MTOC polarization selectively interferes with the delivery of IL-12–containing vesicles at the IS and impairs the activation of the IFN-γ response in CD8+ T cells.

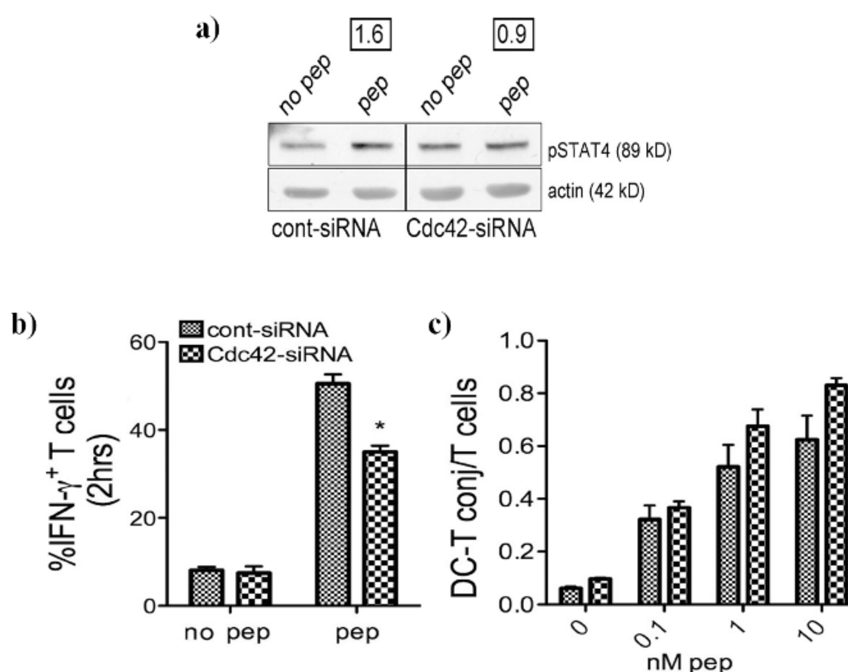

Figure 10. Cdc42 depletion in DCs impaired early IL-12–dependent events in T cells. a) Control and Cdc42-silenced cells activated by TLRs agonist and loaded (peptide) or not loaded (no peptide) with 10 nM of class I peptide were mixed with OT-I cells for 30 min and lysed to analyze the levels of activated STAT4 by W.B. Numbers indicate the intensity values (ratio pSTAT4 peptide/pSTAT4 no peptide normalized for actin levels) obtained from density scans. **b)** Neosynthesis of IFN-γ in T cells engaged in synapse with control or Cdc42- silenced cells. DCs treated as in A were incubated for 2 h, fixed, and analysed for intracellular IFN-γ in T cells engaged in synapse (gated on DC–T cell doublets) by FACS. Values represent average of three independent experiments (*, P = 0.0133). **c)** Conjugate formation is not affected by Cdc4 silencing. 10^5 control or Cdc42- silenced DCs were loaded with graded doses of peptide as indicated, labeled with SNARF and mixed with 10^5 CFSE labeled OT-I for 20 min at 37°C. The number of DC–T cell doublets was measured by FACS gating on the double-positive events and normalized to the number of T cells. Values shown are mean ± SD of three experiments.

As IL-12 produced by DCs regulates survival of primed T cells, we finally assessed the fate of T cells at later time points. DCs transfected with plasmids coding for unrelated-siRNA (control DCs) or for Cdc42 siRNA were stimulated by TLR agonist, loaded with graded peptide doses, and mixed with OT-I cells that had been prelabeled with the vital dye CFSE to follow cell division. At day 3, cells were analyzed for cell division profile, total number of cells, and secretion of IFN-γ. OT-I cells entered division starting at 1 pM and underwent a maximum of 5 cycles. The number of OT-I cells in culture with control DCs increased steadily reaching a maximum at 100 pM. In sharp contrast, DCs treated with Cdc42-siRNA induced OT-I cells to enter division (we observed one cycle of delay only at the 10 pM dose), but divided cells did not accumulate as shown by the marked reduction in the total number of cells (Fig. 11a). This indicates that Cdc42-silenced cells are not competent to promote survival of primed T cells. T cells activated by Cdc42- depleted DCs show an intrinsic per-cell reduction in the percentage of IFN-γ–positive cells at day 3 after priming, which is reflected in the lower levels of IFN-γ in day 3 cell culture supernatant (Fig. 11b). A similar reduction in IFN-γ levels was obtained using DCs expressing the dominant-negative mutant of Cdc42 (Cdc42N17) proving that inhibition of Cdc42- mediated MTOC polarization by two distinct approaches has the same impact on T cell functions (Fig. 11c). Moreover, by coexpressing a rescue plasmid insensitive to Cdc42 silencing, we partially rescued the levels of IFN-γ secreted by T cells stimulated by silenced cells, proving the specificity of the Cdc42 effect (Fig. 11d). Collectively, we conclude that synapse formation and Cdc42-mediated polarization of intracellular stores f IL-12 is required for the acquisition of effector functions by CD8+ naive T cells

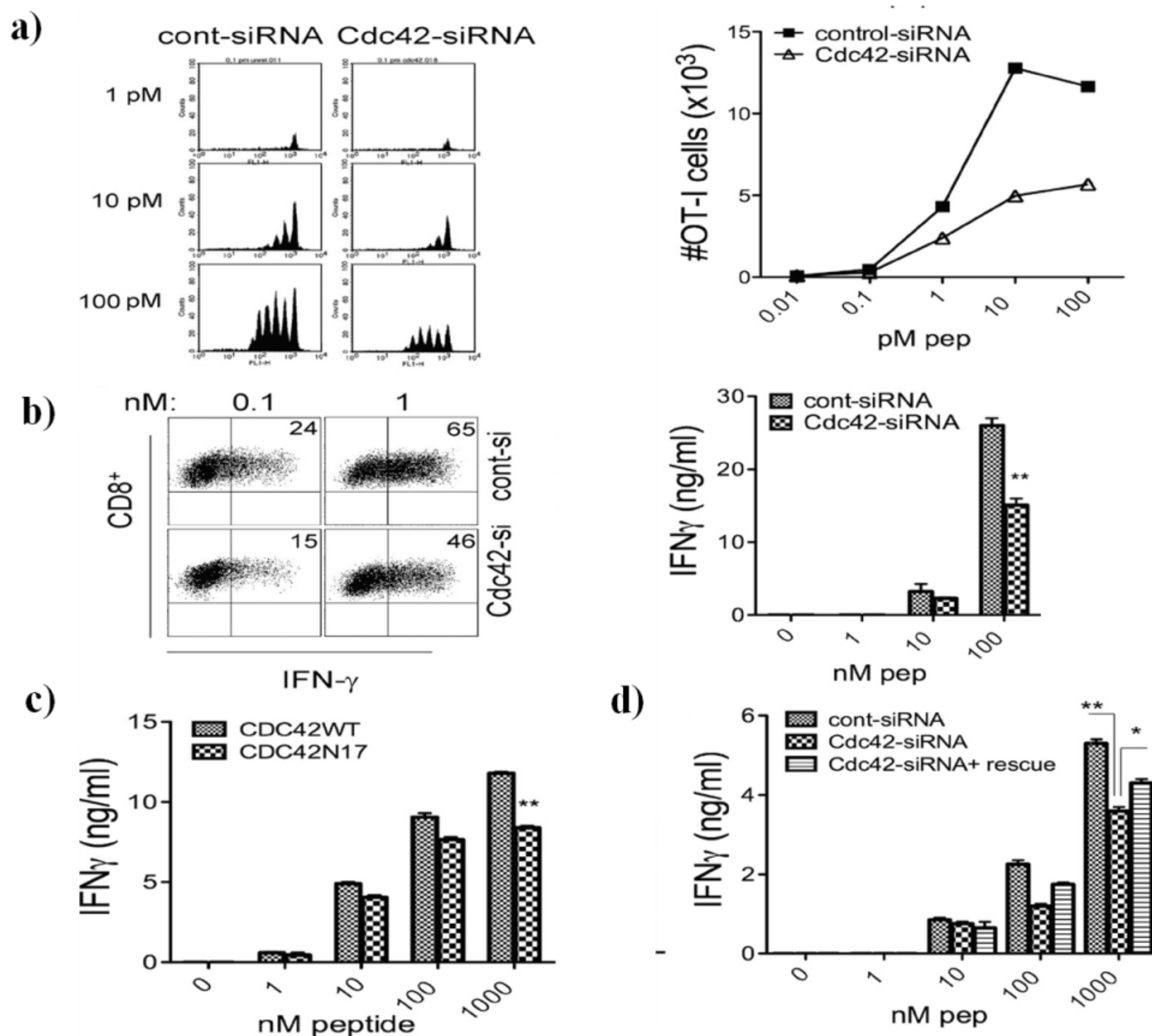

Figure 11. Cdc42 depletion in DCs impairs late IL-12–dependent events in T cells. a) T cells proliferation and survival are affected by Cdc42 silencing in DCs. 10^4 control (control-siRNA) or Cdc42-depleted (Cdc42-siRNA) DCs were TLR stimulated, **l**oaded with graded doses of peptide, and mixed with OVA-specific OT-I cells that had been prelabeled with CFSE. (left) Day 3 CFSE dilution profiles at the indicated doses of peptide. (right) The plot shows the total number of cells recovered at day 3. One of three independent experiments with similar results is shown. **b)** Dot plots show the percentage of IFN-γ–producing cells at day 3 after priming, when T cells were primed using control or Cdc42-depleted DCs. Data show one representative of two experiments. Cell culture supernatants were harvested at day 3, and the levels of IFN-γ were measured by ELISA (values are mean ± SD of three independent experiments (**, P = 0.001). IFN-γ levels secreted by T cells are inhibited by DCs expressing dominant-negative Cdc42. Cdc42WT or the dominant negative Cdc42N17 were stimulated as in D and mixed with OT-I. Bars show the levels of IFN-γ in day 3 cell culture supernatants. Data are the means ± SD of three independent experiments (**, P = 0.0017). **c)** IFN-γ levels secreted by T cells are inhibited by DCs expressing dominant-negative Cdc42. Cdc42WT or the dominant negative Cdc42N17 were stimulated as in **b)** and mixed with OT-I. Bars show the levels of IFN-γ in day 3 cell culture supernatants. Data are the means ± SD of three independent experiments (**, P = 0.0017). **d)** Expression of a rescue construct partially restores IFN-γ levels. DCs were cotransfected with control or Cdc42-specific siRNA, plus a plasmid encoding a Cdc42 resistant to silencing (rescue). Bars shows values of IFN-γ in day 3 cell culture supernatants from 2 independent experiments (means ± SD; *, P = 0.0385, **, P = 0.0068).

3.2. Further into polarity-Disc Large protein (Dlg1)

We highlighted a new mechanism operating in DCs during antigen presentation *i.e.* the polarization of MTOC toward T cell to optimize T cell priming. To increase our understanding of DCs polarity we continued to investigate the molecules involved in establishing polarized state in DCs. Disc Large protein (Dlg), a member of Scrib polarity complex, is classified within one of the five modules ensuring the polarity-dependent functions in T cells (Krummel and Macara, 2006). Specifically, Dlg1 polarizes toward the synapse in T cells where it associates with other molecules (TCRζ and Cbl) shown essential for the proper synapse signaling (Xavier et al., 2004). It is one of the factors ensuring appropriate asymmetrical division of T cells in synapse with APC (Oliaro et al., 2010). Dlg clusters at the uropod of migrating T cells (Ludford-Menting et al., 2005), an event that is most likely important for the proper T cell locomotion. On the other side, however, questions regarding the nature and the role of polarity proteins in DCs have not been addresses so far. Following our initial insights into how polarity phenomenon (MTOC translocation) can regulate the function of DCs, we asked whether Dlg participates in polarity-related processes in DCs.

3.2.1. Dlg1 localizes mainly in the membrane area in immature DCs

In mammalian cells, Dlg is present in four isoforms (Dlg1-Dlg4). Out of four isoforms, Dlg1 is ubiquitously present within most of the tissues studied (Assemat et al., 2008) and its role in ensuring polarization events has been documented in T cells (Rebeaud et al., 2007; Round et al., 2005; Xavier et al., 2004), as well as others (Etienne-Manneville, 2008; Hawkins and Russell, 2008). As there were no data regarding Dlg1 in DCs, we checked Dlg1 expression pattern in immature DCs, by RT-PCR. As shown on the gel, Dlg1 was expressed in immature DCs (Fig12a). In addition, we assessed the protein levels of Dlg1 in immature DCs by probing the cell lysates with anti-Dlg1 antibody. Since in T cells the expression of Dlg1 is very well documented (Hanada et al., 1997; Round et al., 2005), these cells were used in order to understand the levels of Dlg1 expression in DCs. As it can be seen from the representative gel, the expression of Dlg1 is much higher in immature DCs than in T cells (Fig12b).

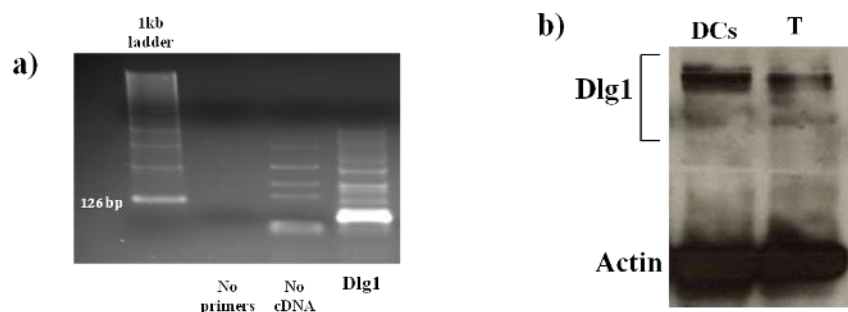

Figure 12. Assessing Dlg1 expression in immature DCs. a) RT-PCR analysis of Dlg1 mRNA levels in immature DCs **b)** Western blot analysis of Dlg1 expression in immature DCs. Total cell lysates of DCs or T cells were probed with the antibody recognizing Dlg1. T cell lysates were used as a positive control.

Having established that Dlg1 is abundantly expressed in DCs, we went on to assess the exact localization of Dlg1 in immature DCs. This was done by labeling immature DCs with the antibody against Dlg1 and co-staining with phalloidin (to detect F-actin) (Fig13, *upper panel*). As shown in the confocal planes, Dlg1 is present in the form of discrete punta in F-actin rich areas in immature DCs. Since F-actin mainly clusters subcortically in immature DC (Kobayashi et al., 2001), this suggested that Dlg1 localizes underneath the membrane zone in immature DCs. This was further confirmed by revealing the shape of immature DCs, stained with the antibody against Dlg1, in transmitted light. Confocal analysis of intracellular distribution of Dlg1 clearly shows that all Dlg1 signal is found at cellular periphery (Fig13, *lower panel*).

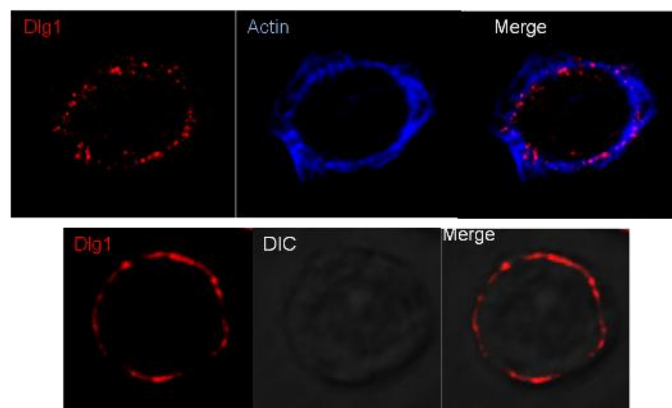

Figure 13. Assessing Dlg1 localization in immature DCs. Representative images of immature DCs labeled with anti-Dlg1 antibody (red) and phalloidin (blue) (*upper panel*) or looked under the transmitted light (*lower panel*).

Therefore, from the previous figures it could be concluded that Dlg1 is expressed in immature DCs where it is mainly localized in the membrane area.

3.2.2. Dlg1 undergoes relocalization from membrane toward the protrusions in mature DCs

It is well established that DCs undergo morphological changes upon TLR stimulation, going from small rounded immature cells to larger cells with increased number of membrane protrusions *i.e.* podosomes, filopodia and lamellopodia (Burns et al., 2001). These morphological structures are the outcome of the actin cytoskeletal remodeling associated with establishment of the migrational phenotype (de Noronha et al., 2005). Furthermore, a vast array of polarity proteins accompanies these changes and contributes to the migrational capacity of various cell types (Etienne-Manneville et al., 2005; Ludford-Menting et al., 2005). We asked whether Dlg1 undergoes relocalization upon DCs maturation. For that purpose, we left DCs untreated or stimulated them for 1h and 5h. After the end of the treatment, we plated DCs on the fibronectin-coated slides and stained them with the antibody against Dlg1. As seen in transmitted light, at 1h post TLR stimulation Dlg1 was mainly clustered at the membrane protrusions, although some Dlg1 staining was also evident on the opposite side. This pattern sharpened even more as 5h post TLR stimulation Dlg1 was localized exclusively in the membrane protrusions (Fig14). Hence, we conclude that Dlg1 undergoes translocation from subcortical regions towards membrane protrusions in TLR stimulated DCs.

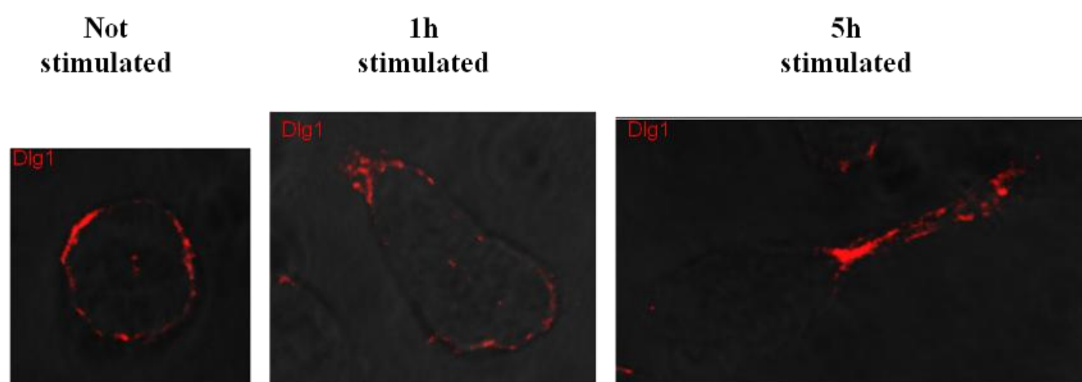

Figure 14. Changes in Dlg1 localization in DCs upon TRL stimulation. Representative images demonstrating Dlg1 localization in TLR stimulated DCs. DCs were stimulated for the indicated periods with a mix of TLR agonists. The cells were then labeled with the antibody against Dlg1 (red) and analysed under transmitted light.

TLR stimulation induces a complete alteration of transcription program in DCs as it upregulates the expression of chemokines receptors (CCR2, CCR5 and CCR7) and adhesion molecules (integrin α4) essential to enable cell locomotion toward lymphoid tissues (Akira, 2001; Puig-Kroger et al., 2000; Sallusto et al., 1998). We wondered whether TLR stimulation induces an increase in Dlg1 expression in DCs. For that purpose, DCs were stimulated with the mix of CpG and LPS for 2h, 4h and

6h. At the end of each period, the cells were lysed and the expression of Dlg1 was checked both by qRT-PCR and WB (Fig 15a and b). As it can be seen from both experiments, Dlg1 does not undergo *de novo* expression post TLR stimulation.

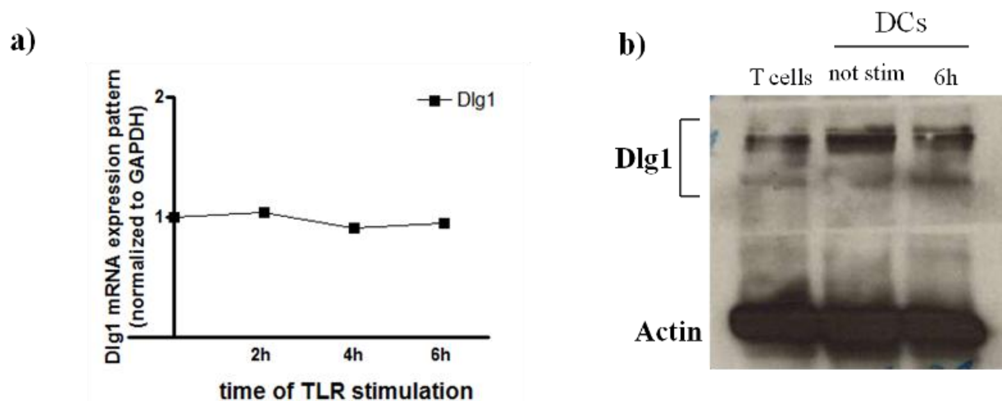

Figure 15. Dlg1 expression profile in DCs post TLR stimulation. DCs were stimulated with the mix of CpG and LPS for the indicated periods. The cells were then lysed, total RNA was retro-transcribed and amplified by qRT-PCR using Dlg1-specific primers (**a**); total proteome separated by gel-electrophoresis and blotted with anti-Dlg1 antibody (**b**).

Together these data indicate that TLR stimulation does not induce *de nov*o expression of Dlg1, but does induce relocalization of the available pool of Dlg1 into membrane protrusions.

3.2.3. *Dlg1 is phosphorilated upon TLR stimulation*

Dlg activation status was shown to be regulated by phosphorilation in most of the mammalian cells investigated in response to stimuli (Koh et al., 1999; Massimi et al., 2006; Narayan et al., 2009), including T cells (Hanada et al., 1997; Koh et al., 1999)We thus asked whether Dlg1 phosphorilation status changes following TLR stimulation in DCs. It has been previously documented that phosphorilation events cause mobility shifts in Dlg, visible in the appearance of several higher, closely spaced migrating bands in the western blot (Mantovani et al., 2001). Therefore, we run the gels of DCs stimulated for different periods to analyze the mobility pattern of Dlg1 in resting and stimulated DCs. In not stimulated DCs two major Dlg1 bands are detected. However, at 1h post TLR stimulation only the slower migrating band was visible plus a smear of higher bands. This pattern was maintained also at 3h whilst the disappearance of higher bands and simultaneous appearance of very weak lower bands was observed at 5h post TLR stimulation (Fig 16a). Treating DCs with phosphatase following TLR stimulation changed the mobility of the higher molecular weight forms demonstrating that they are indeed phosphorilated (not shown). This experiment clearly demonstrated that Dlg1 becomes hyper-

phosphorilated in response to TLR stimulation. Furthermore, it indicated that this change occurs at early time points post TLR stimulation. In a shorter kinetic experiment we established that phosphorilation of Dlg1 takes place as early as 30 min post TLR stimulation (Fig 16b).

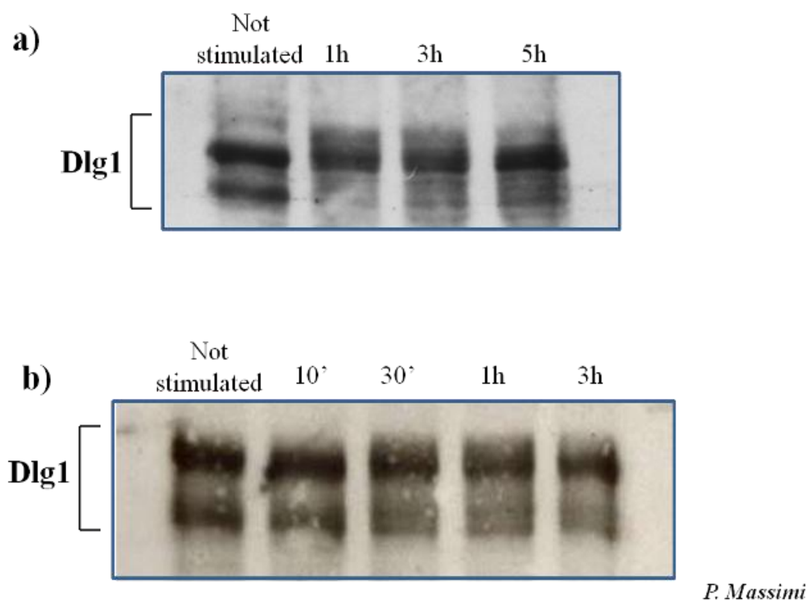

Figure 16. Dlg1 phosphorilation pattern in DCs post TLR stimulation. DCs were stimulated with TLR agonists for indicated periods of time. Total cell lysates were separated by SDS-PAGE and probed with anti-Dlg1antibody **a)** Long-term kinetics of Dlg1 phosphorilation profile in DCs. **b)** Short-term kinetics of Dlg1 phosphorilation profile in DCs.

From our observations regarding Dlg1 expression in DCs prior to TLR stimulation we were able to conclude that Dlg1 is indeed expressed in immature DCs, where it localizes mainly in the membrane area. TLR stimulation does not induce Dlg1 up regulation on the gene level, but does induce changes in its phosphorilation status. Moreover, TLR stimulation of DCs is coupled with Dlg1 relocalization toward the membrane protrusions.

Thus, we highlighted a novel link between TLR stimulation and dynamics of polarity proteins in DCs. This was not further pursued in this work but it would be of great interest to understand the pathways that link TLR stimulation and polarity proteins dynamics to post-transcriptional modifications of Dlg1.

3.2.4. Dlg1 polarizes toward the synapse in TLR and antigen dependent manner

Following TLR stimulation, DCs undergo the process of maturation in which they lose the capacity to recognize antigens, but acquire the capacity to present these antigens to T cells within the immunological synapse (IS) (Benvenuti et al., 2004b; Celli et al., 2008; Hugues et al., 2004). IS is a place of physical contact between APC (DC) and T cell where these cells share the signals regarding the nature of the pathogen. The outcome of such association is the activation of adaptive immune response characterized by T cell proliferation and differentiation. Within the IS, change in membrane receptors and intracellular proteins takes place on both sides of the IS. Spatial redistribution of signaling components at the IS is driven by remodeling of actin and microtubule cytoskeleton (Al-Alwan et al., 2001a; Benvenuti et al., 2004a; Krummel and Macara, 2006). Recently, the role of polarity proteins in establishing correct positioning of molecules at the IS has been unveiled in T cells. Thus, it has been shown that the presence of Scrib is necessary for the synapse functioning, as knockout T cells show a striking defect in their ability to form stable conjugates (Ludford-Menting et al., 2005). Polarization of Dlg1 at the synapse was shown to induce actin and TCR clustering, as well as autocrine cytokine (IL-2 and IFN-γ) production in CD8+ T cells (Round et al., 2005). PKCζ-dependent Par1 clustering at the synapse is important for the MTOC translocation toward the synapse in T cells (Lin et al., 2009).

From the previous experiments we were able to see that Dlg1 is expressed in DCs and relocalizes toward membrane protrusions upon TLR signaling. Given the fact that the formation of the functional synapse is dependent on the events on both side of the synapse, we asked whether Dlg1 might have a role in synapse stabilization and/or maintenance on DCs side. In order to answer to that question, we tracked distribution of Dlg1 in DCs during synapse formation. Specifically, DCs were stimulated with TLR agonists for 4h in the presence of antigen. CFSE labeled T cells were added for the total duration of 5min, 15min or 30min. At the end of each period, the cells were labeled with the antibody recognizing Dlg1 and its position in DCs relative to the synapse was assessed. The representative confocal images of cells with polarized *vs.* not polarized Dlg1 are given (Fig17, *upper panel*). As it can be seen from the corresponding graph (Fig 17, *lower panel*), there is an immediate Dlg1 clustering toward the synapse area as early as 5min post synapse formation (40%), the phenomenon that further increased at 15min (53.8%) but remained the same at 30min (51.4%) post synapse formation. To understand whether Dlg1 clustering is triggered by antigen recognition we performed a parallel experiment using DCs not loaded with antigen. At 5min there were virtually no synapses while the number of synapses at 30min was very low (less than 20), which made it hard to gain a statistically

significant number of the events. Still, Dlg1 was present at the synapse area 30min post synapse formation with a lower magnitude then in the corresponding condition in the presence of antigen (37.5%, not shown).

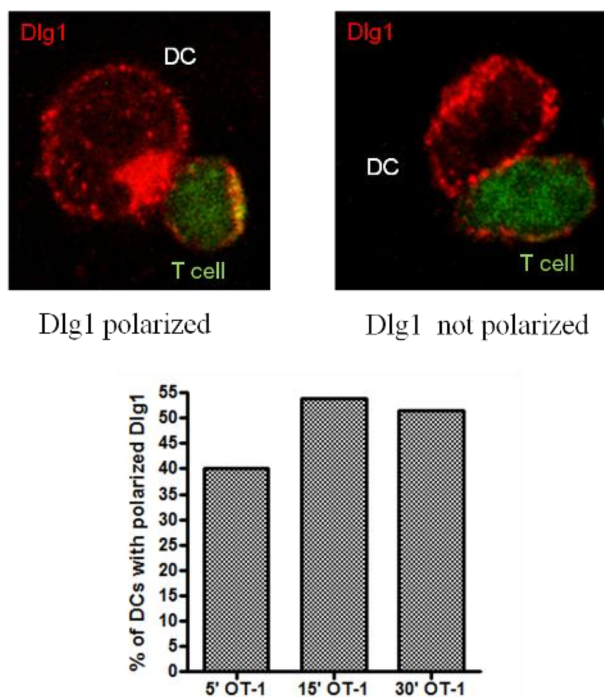

Figure 17. Dlg1 localization in DCs during synapse formation. *Upper panel*: Representative images of Dlg1 localization within DCs in synapse with T cell. *Lower panel*: Dlg1 distribution relative to synapse in TLR-stimulated DCs. DCs were stimulated for 4h in the presence of TLR agonists and 10nM peptide. The cells were then left to form the synapses with CFSE stained-T cells (5min, 15min and 30min) on fibronectin coated slides. Afterwards, the cells were stained with the antibody against Dlg1. The distribution of Dlg1 was then calculated as described in *Materials and Methods,* and the results were summarized in the graph. A representative graph of 2 independent experiments is shown.

Dlg1 presence in the synapse area in unspecific manner implies that its clustering is not initiated by TCR-specific signaling but it is, however, further strengthened with the addition of antigen. The role of integrin-mediated polarization events is not surprising as their involvement has been shown in other models system as well. In migrating astrocytes, for instance, it has been demonstrated that integrin-mediated clustering of Cdc42 at the leading edge induces further recruitment of Par6-aPKCζ (Etienne-Manneville and Hall, 2001) as well as Dlg and APC (Etienne-Manneville et al., 2005) . In DCs, the engagement of integin (LFA) molecules on DCs side by ICAM-1 and ICAM-3 on T cells side was shown to induce even higher expression of MHC molecule (de la Fuente et al., 2005). Hence, one likely hypothesis is that the initial interaction between integrins induces stronger TCR signaling; this, in turn, causes further affinity changes of integrins, amplification of the signal and stronger Dlg1 clustering. We investigated whether Dlg1 polarization coincides with polarization of MTOC in TLR-stimulated DCs

engaged in antigen-specific synapse with T cells. By co-labeling cells with anti-Dlg1 and anti-α tubulin antibodies, we found a correlation between Dlg1 and MTOC polarization in most of the synapses checked (in about 30 synapses). (Fig18). These results suggest that both Dlg and MTOC follow the same kinetics of polarization toward the synapse in TLR stimulated DCs. Physical and functional connection between Dlg and MTOC has been reported before in T cells (Hanada et al., 1997; Lasserre et al., 2010); however further in-depth experiments are needed in order to assess their putative interaction in DCs during IS formation.

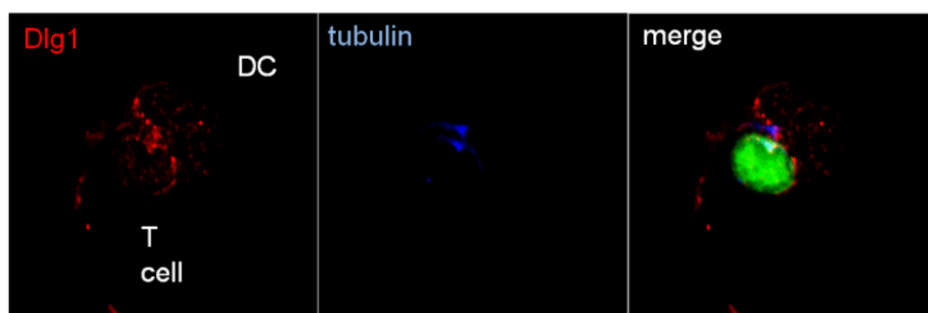

Figure 18. Dlg1 and MTOC are enriched at DC-T cell interface. A representative image of Dlg1 (red) and MTOC (blue) localization in DCs in synapse with CFSE-stained T cell.

Together these results indicate that polarity in DCs is defined by reorientation of MTOC and an increase in Dlg1 clustering in synapse following TCR signaling

3.3. Function of Dlg1 in DCs

Our findings show that Dlg1 undergoes redistribution toward membrane protrusions in mature DCs and its clustering at the IS when DCs interact with T cells. To investigate the functional significance of Dlg1 redistribution in DCs, we set up a protocol to deplete its expression. For that purpose we applied a siRNA-based Dlg1 depletion of DCs. In short, DCs were nucleotransfected with siRNA against Dlg1 at day 5 of their differentiation. 48h post transfection, the cells were collected, lysed and the efficiency of silencing was checked both by WB and qRT-PCR (Fig19).

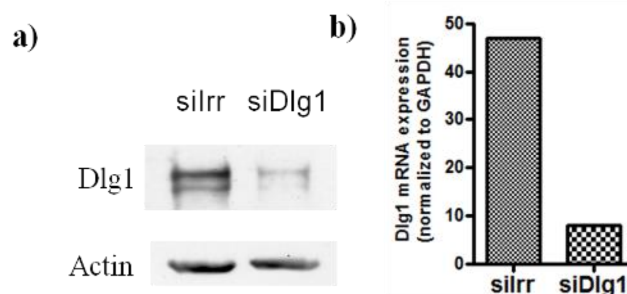

Figure 19. Checking the efficiency of silencing by western blot and qRT-PCR. DCs, transfected with siRNA against Dlg1, were assessed for the efficiency of silencing by probing the cells lysates with the antibody against Dlg1 (*left panel*) or by evaluating the levels of Dlg1 mRNA by qRT-PCR (*right panel*). The level of Dlg1 mRNA in transfected DCs was measured against the expression of GAPDH.

In order to make sure that DCs do not experience some intrinsic defects due to loss of Dlg1 we have checked whether they retain their cytokine-producing and maturation capacity. Dlg1-depleted DCs (and Control DCs) were TLR stimulated for 4-6h. Afterwards, the levels of IL-12 were assessed from the culture supernatants. Simultaneously, we measured the upregulation of maturation markers (CD40, MHC and CD86) on the surface of DCs by FACS. Dlg1 depletion did not affect DCs' capacity to produce IL-12 or their capacity to up-regulate the maturation markers expression post-TLR stimulation (Fig20a and b).

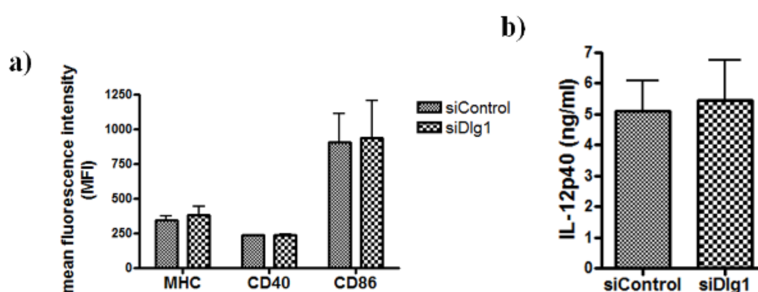

Figure 20. Dlg1 depletion does not have an effect on cytokine secretion (a) nor maturation capacity (b) of DC. Dlg1-depleted (or Control DCs) were TLR-stimulated for 4-6h. The concentration of IL-12 was measured from the supernatants by ELISA (a). Simultaneously, the cells were fluorescently stained for CD40, MHCI and CD86 expression. The percentage of fluorescent cells was measured by FACS (b). The values represented in the graphs are the means ± SEM of 3 independent experiments.

3.3.1. Dlg1 and migration

To evaluate a possible role of Dlg1 in DCs migratory capacity, we performed at first an *in vitro* migration assay using transwell. Two cytokines were used for this purpose, CCL19 and CCL21, whose role as the major inducers of DCs migration was well documented (Alvarez et al., 2008; Dieu et

al., 1998). In order to test whether DCs respond to chemokine gradient in this system, we first performed a transwell assay with not transfected wtDCs. In short, LPS-stimulated DCs were placed into the inserts and left to migrate 3h at 37C. As expected, wtDCs cells migration toward CCL19 and CCL21 was greatly enhanced with the addition of chemokines (75% cells in the case of CCL19; 100% cells in the case of CCL21) (Fig21a). The same assay was applied to DCs treated with control or Dlg1 specific siRNA. First, we noted that the number of migrated DCs was much lower comparing to the corresponding conditions in not transfected wtDCs, regardless of the siRNA used (there was a 75% and 85% of reduction in percentage of migrated cells with CCL19 and CCL21, respectively). We reasoned that this could probably be due to the effect that transfection might have on DCs. Nevertheless, transfected DCs did react to chemokine gradient by the increase in migration, relative to the situation when chemokines were not used. However, the difference in the migratory capacity between Dlg1-depleted DCs and Control DCs was not observed in these settings (Fig 21b). Hence, we conclude that Dlg1 does not have an effect on the DCs responsiveness to chemokine gradient *in vitro.*

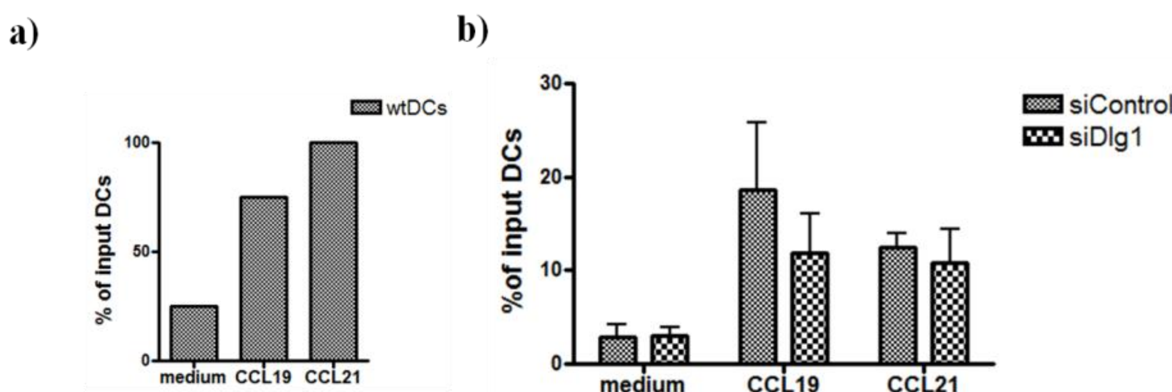

Figure 21. Assessing the role of Dlg1 in DCs responsiveness to CCL19 and CCL21. a) CCL19 and CCL21 induce wtDCs migration. **b)** Dlg1 depleted DCs do not experience a defect in migratory capacity in response to chemokines. DCs (both a and b) were LPS stimulated for 3h at 37C. These cells were then subjected to the difference in the chemokines (CCL19 or CCL21) concentration for 3h at 37C in an *in vitro* transwell assay. The number of DCs at the lower well was then counted and the number was expressed as the percentage (%) of total cells. The data on the graph represent the means±SEM of 5 independent experiments.

To fully assess whether Dlg1 has a role in migratory capacity of DCs, Dlg1-depleted DCs were tested for their capacity to migrate *in vivo*. In short, 48h post Dlg1-depletion CFSE stained DCs were injected into the footpad of the recipient mice. The cells were left to migrate for 24h upon which the draining lymph nodes were taken. The cells were then visualized by FACS and the number of DCs reaching the lymph node was expressed as the percentage of the total injected population. As it can be seen from the graph (Fig22), the percentage of the cells that reached the draining lymph node was the

same in both Control-DCs and Dlg1-depleted DCs conditions. Hence, we conclude that Dlg1-depletion in DCs does not compromise their migration capacity *in vivo.*

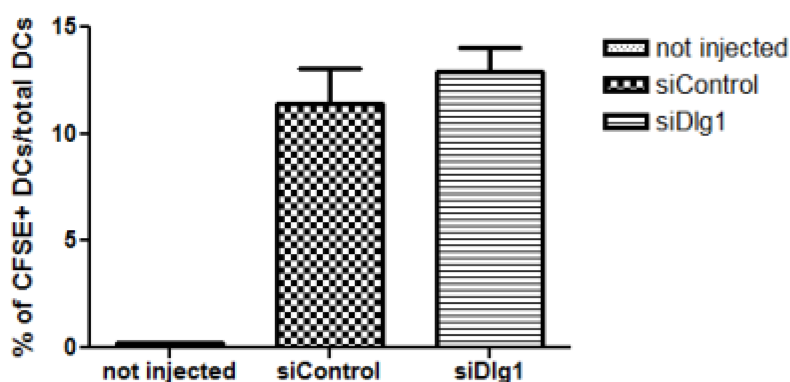

Figure 22. Assessing the role of Dlg1 in DCs migratory capacity *in* vivo. Dlg1-depleted (or Control) DCs were collected 48h post transfection and stained with unspecific dye (CFSE). The cells were then injected into the footpad of the recipient mice and their migratory capacity was evaluated by assessing the percentage of CFSE+ DCs over total DCs population in the proximal lymph node after 24h (see *Materials and Methods).* The values represented on the graphs are mean ± SEM for 2 independent experiments of 4 mice each. The not injected mice were used as the control of the experimental procedure.

In general, our *in vitro* and *in vivo* data regarding the migratory capacity of DCs indicate that Dlg1 does not have a role in this specific function of DCs.

3.3.2. Dlg1 in synapse formation and T cell functioning

We have previously shown that both MTOC and Dlg1 redistribute toward the IS in DCs in antigen specific manner, following TLR stimulation. Moreover, by co-labeling MTOC and Dlg1 in the same DC, we found them associated underneath the plasma membrane in IS. These observations were not unexpected as the link between MTOC and polarity proteins has already been well documented in various cells (Iden and Collard, 2008; Manneville et al., 2010), including T cells (Lin et al., 2009; Quann et al., 2011). All of the above prompted us to ask whether there was a functional connection between MTOC and Dlg1 in DCs in relation to the synapse formation. Having previously established that the highest incidence of MTOC polarization toward the synapse (42%) occurs in the conditions when TLR-stimulated DCs were left in synapse with T cells for 30min in the presence of 10nM peptide (Fig1), we used the same conditions in order to check the incidence of MTOC translocation in Dlg1-depleted DCs. At the end of synapse period, the cells were stained with the antibody against α-tubulin in order to visualize MTOC position. The incidence of MTOC translocation toward the synapse in siControl DCs was 56.3% ±1.820*,* a number that decreased to 39.28%±0.390 in siDlg1 depleted DCs (30% of reduction) (Fig23).

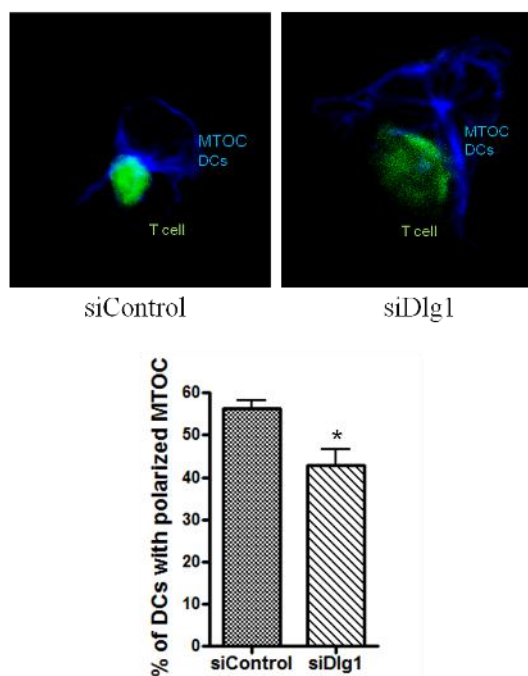

Figure 23. The incidence of MTOC translocation in DCs lacking Dlg1. Dlg1-depleted DCs were stimulated with TLR agonists for 5h in the presence of 10nM peptide. CFSE-labeled T cells were then layered over and left for 30min synapse time. Cells were then labeled with the antibody recognizing α-tubulin (blue) in order to visualize MTOC location. *Upper panel*: Representative images showing MTOC location in siControl and siDlg1 depleted DCs. *Lower panel*: MTOC distribution relative to synapse in Control and Dlg1 depleted DCs. The incidence of MTOC translocation was measured as described in *Materials and Methods*. The values are represented as means±SEM for 2 independent experiments (60-70 synapses counted).

These results demonstrated that Dlg1 depletion has an impact on the incidence of MTOC translocation toward the synapse in TLR-stimulated DCs. Thus Dlg1 is important to establish cell polarity in DCs at the IS by affecting MTOC movement.

3.3.2.1. Assessing the functional consequences of Dlg1 depletion in DCs on T cells functions

3.3.2.1.1. CD69 expression and IL-2 production in T cells stimulated by Dlg1-depleted DCs

MTOC polarization in DCs toward the synapse brings a functional message to T cells, as previously established (Fig4 and Fig5). This was further confirmed by the decrease in the early and late activation events in T cells forming a synapse with DCs in which MTOC polarization has been abrogated (Fig10 and Fig11). Now we have shown that the absence of Dlg1 in DCs had an impact on MTOC translocation toward the synapse. Therefore, in order to fully assess the role of Dlg1 in the process in DCs polarity, we checked whether Dlg1 depletion in DCs affects early and late functional events in T cells.

Some of the early events in T cells activation are CD69 expression and IL-2 production. CD69 is a transmembrane C-type lectin expressed on the surface of T cell early upon activation. IL-2 is a cytokine secreted by the activated T cells that enables clonal expansion of pathogen-activated T cells. CD69 expression and IL-2 production were used as the markers of activation of T cells. In short Dlg1-depleted DCs (or Control DCs) were stimulated for 4-6h with TLR agonists in the presence of graded doses of antigen. The excess of antigen was then washed away and T cells were added. 24h later, the concentration of IL-2 was measured from the co-culture supernatants and, in parallel; T cells were stained for the presence of CD69. Both siControl DCs and siDlg1 depleted DCs induced the expression of CD69 as well as the IL-2 production in T cells, in antigen dose dependent fashion. However, we found no difference in the levels of these activation markers in T cells co-cultured with siControl DCs or siDlg1 depleted DCs (Fig24a). Hence, we conclude that Dlg1 depletion did not affect the early activation of T cells *in vitro*.

We also measured early T cell activation *in vivo*. Dlg1-depleted DCs were loaded with OVA peptide and injected into the footpad of the recipient mice that have previously been adoptively transferred with OT-1 (CD45.1 congenic) cells. 24h later draining lymph nodes were taken and the percentage of OT-1/CD45.1 positive for the expression of CD69 was then assessed. As shown in the Fig24b, there was a slight decrease in the levels of CD69 positive OT-1/CD45.1 cells in mice injected with Dlg1-depleted DCs as compared to mice injected with Control DC; however, this decrease was not statistically significant.

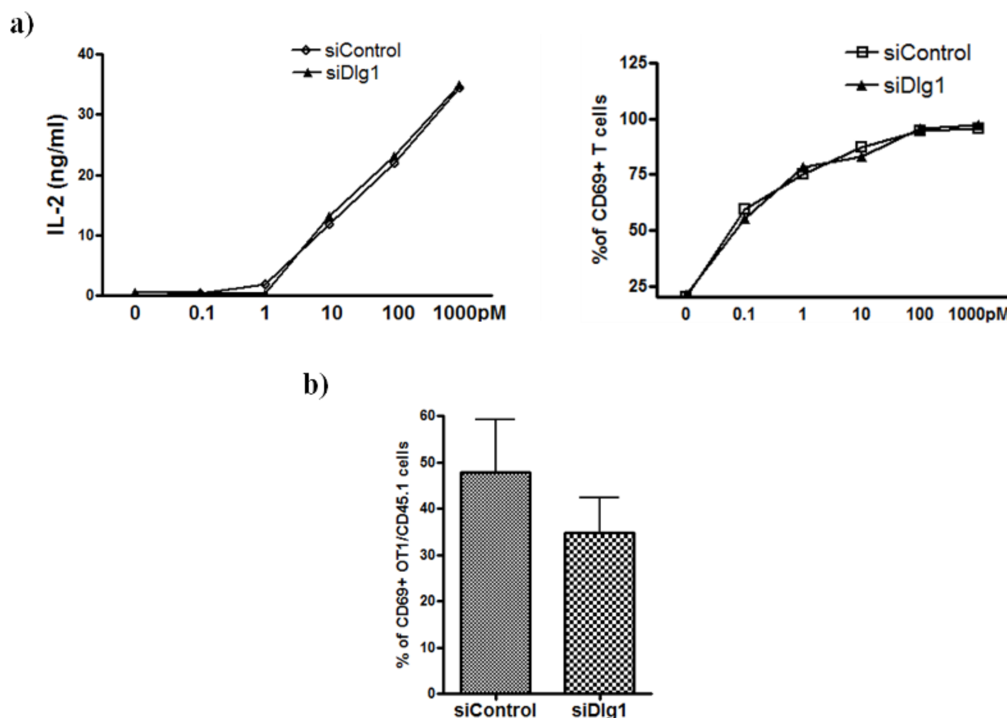

Figure 24. Dlg1 depletion in DCs does not affect early activation of T cells. a) Dlg1 depletion in DCs does not affect CD69 expression and IL-2 production in T cells *in vitro.* DCs were stimulated for 3h with the mix of TLR agonists in the presence of graded doses of peptide, washed and mixed to T cells. 24h later, concentration of IL-2 was determined by ELISA from the co-culture supernatants (left); concomitantly, T cells were stained for the CD69 expression, which was then visualized by FACS (right). One of the three independent experiments with similar results are shown **b)** Dlg1 depletion in DCs does not affect CD69 expression in T cells, *in vivo.* Dlg1-depleted DCs, previously pulsed 3h with peptide, were injected into the footpad of the recipient mice. OT-1 (CD45.1 congenic) cells were adoptively transferred the day before. 24h post DCs injection, poplitheal lymph nodes were taken and the percentage of OT-1/CD45.1 cells positive for CD69 marker was obtained by FACS. The values are represented as means ± SEM of three independent experiments.

Therefore, we conclude that Dlg1 depletion in DCs does not have an effect on the early activation events in T cells, *in vitro* and *in vivo.*

3.3.2.1.2. INF-γ production in T cells stimulated by Dlg1-depleted DCs

Within the adaptive immune response, T cells undergo the process of differentiation in which they acquire lineage-specific functional and phenotypical characteristics. It is becoming clear that T cell differentiation toward different T cell fates depends on the events that occur very early during T cell activation (Celli et al., 2008; Clark et al., 2011). Our previous data suggest that indeed polarization of MTOC at the IS cause STAT4 phosphorilation in T cells and early production of IFN-γ protein in T cells engaged in synapse with polarized DCs. Here to better understand the relevance of DCs polarity in T cell programming, we set up an assay to quantitatively measure induction of IFN-γ genes very early upon synapse formation, by qRT-PCR.TLR-stimulated wtDCs (5h) were loaded or not loaded with

OVA class I peptide and mixed to T cells for 1h. As controls we used DCs alone, not stimulated (*immature* DCs) or stimulated with TLR agonists (*mature* DCs), and T cells alone, not pulsed (*T cells*) or pulsed with peptide (*T+peptide*). T cells mixed with mature DCs in the presence of petide produced much higher (7x) levels of IFN-γ than T cell incubated with mature DCs in the absence of peptide. We observed a slight induction also when mature DCs were mixed to T cells in the absence of peptide as compared to mature DCs or T cells alone, indicating that simply mixing the two cell types induce a response, which was nevertheless much lower than the one induced in the presence of peptide. The levels of IFN-γ produced by immature DCs alone and T cell alone (in the presence or absence of peptide) were negligible (Fig 25a). This indicates that formation of antigen-specific DC-T conjugates with mature DCs induce a very early activation of IFN-γ gene transcription in T cells. Although we cannot formally exclude that formation of antigen-specific synapse induces activation of IFN-γ genes also in DCs, it is likely that most of the signal comes from T cell activation. This assay is, therefore, a robust measure of signaling events that are induced very early upon synapse formation in T cells. We used this assay to evaluate the effect of Dlg1 depletion in DCs on early T cell programming at the IS. Specifically, IFN-γ production was measured in the conditions when Control DCs or Dlg1 depleted DCs were mixed to T cells, in the presence or absence of peptide. As shown in Fig 25b, the levels of IFN-γ transcripts were very low in the absence of peptide. IFN-γ genes expression was pretty enhanced in the presence of peptide both in Control and Dlg1-depleted conditions. However, the level of IFN-γ was significantly lower in T cells in synapse with Dlg1 depleted DCs (32% reduction). Collectively, these results led us to conclude that Dlg1 depletion in DCs abrogated the synapse-specific signaling responsible for the early activation of IFN-γ gene transcription in T cells.

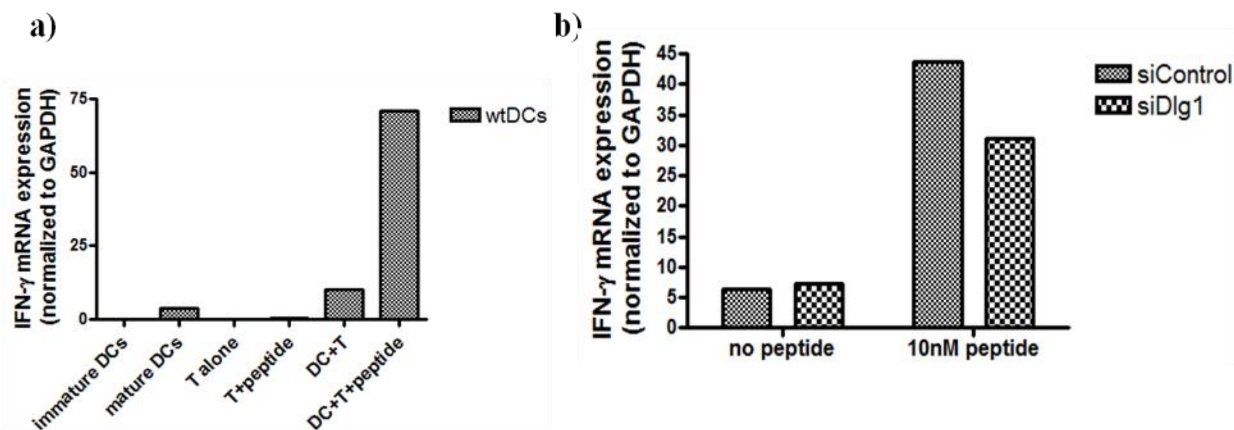

Figure 25. Dlg1 depletion in DCs impairs synapse-dependent early IFN-γ production in T cells. a) Synapse specific events at DC-T cell interface enhance IFN-γ mRNA production in T cells. wtDCs were stimulated with TLR agonists for 5h in the presence or absence of 10nM peptide, washed and mixed to T cells for the total time of 1h. The cells were then lysed and IFN-γ mRNA concentrations were determined by qRT-PCR. DCs alone not stimulated (*immature DCs)* or stimulated with TLR agonist (*mature* DCs) and T cells alone not pulsed (*T* alone) or pulsed with peptide (*T*+peptide) were used as the controls. **b)** Dlg1 depletion in DCs causes a decrease in synapse specific IFN-γ mRNA production in T cells. Dlg1-depleted DCs (or Control DCs) were treated in the same manner as in **a)**. The levels of IFN-γ mRNA were assessed by qRT-PCR. Representative graphs of 2 independent experiments are shown.

Impairment in IFN-γ production at early stages was also reflected at later time points. Specifically, Dlg1-depleted DCs (or Control DCs) were stimulated with TLR agonists for 5h in the presence of graded doses of peptide. T cells are added and the level of IFN-γ was assessed from the supernatants 3 days later. (Fig26). The level of IFN-γ increased gradually with the addition of peptide when both Dlg1 depleted DCs and Control DCs were used. However, the levels of IFN-γ produced by T cells in synapse with Dlg1-depleted DCs were much lower in all peptide doses tested. The highest reduction (54%) was noted when synapses were formed in the presence of 50nM.

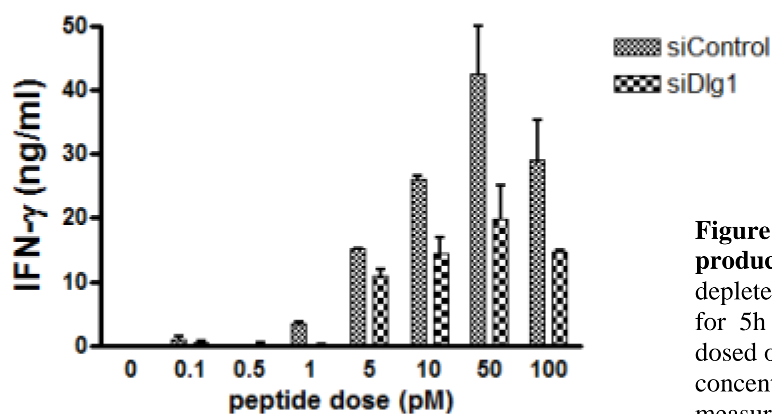

Figure 26. Dlg1 depletion in DCs impairs IFN-γ production in T cells at later time points. Dlg1 depleted DCs (or Control DCs), previously stimulated for 5h with TLR agonists in the presence of graded dosed of peptide, were mixed to T cells. 3 days later, the concentration of IFN-γ in the supernatants was measured by ELISA. The values are represented as means ± SEM of 2 independent experiments.

Finally, we tested the effect of Dlg1 depletion in DCs on their ability to activate T cells *in vivo.* Pilot experiments were performed at first using DCs transfected with control siRNA to establish the conditions of priming *in vivo* using transfected DCs. We identified $3x10^5$ DCs, 2M of T cells and 0.5nM of peptide as optimal conditions to measure T cell priming *in vivo.* Thus, we loaded $3x10^5$ of Dlg1 depleted DCs (or Control DCs) with 0.5nM of OVA peptide and injected them into host mice. The mice were previously adoptively transferred with 2M of CFSE-labeled OT-1 cells. At day 3 following DCs injection, lymph nodes proximal to the injection site were harvested and proliferative capacity in terms of T cell expansion of transferred OT-1 cells was measured. As shown in Fig27a the ratio between transferred OT-1 cells over the endogenous CD8+ T population was equivalent when the mice were immunized with either Dlg1 depleted DCs or Control DCs. This suggested that Dlg1-depletion in DCs did not affect priming *in vivo*. Furthermore, the analysis of CFSE dilution profile showed that transferred OT-1 cells underwent the same number of cell cycles (>7 cycles) and with the same kinetics when mice were immunized with either Dlg1-depleted DCs or Control DCs (not shown). Together, these data suggest that Dlg1 depletion in DCs does not affect their ability to prime T cells *in vivo*.

To assess the acquisition of effector functions in OT-1 cells primed by Control DCs or Dlg1-depleted DCs, we performed *ex vivo* restimulation and intracellular IFN-γ staining of transferred OT-1 (CD45.1 congenic) cells 3 days after DCs immunization. Results presented in Fig27b show there was no difference in the intracellular IFN-γ content of OT-1 cells primed either by Control DCs or Dlg1 depleted DCs. Thus we conclude that Dlg1 depletion in DCs does not affect the capacity of these cells to induce differentiation of T cells *in vivo*.

Collectively, the functional data regarding the role of Dlg1 in DCs in synapse with T cells demonstrated that Dlg1 is important for the early and late induction of IFN-γ production *in vitro.* However, Dlg1 depletion in DCs did not compromise their capacity to prime T cells *in vivo.* There may be a few possibilities for the discrepancy between the data regarding T cell activation by Dlg1 depleted DCs *in vitro* and *in vivo.* Since transfection procedure itself affects DCs vitality, we reasoned that the professional phagocytes (DCs and macrophages) in recipient mice may take up and process the proportion of transfected cells that are dying and present OVA peptide themselves; thus abolishing potential difference in antigen presentation by Control *vs.* Dlg-depleted DCs. Another possibility is that the high peptide dose used to induce priming by transfected DCs may mask the putative difference in priming capacity of Dlg1 depleted DCs *vs.*Control DCs.

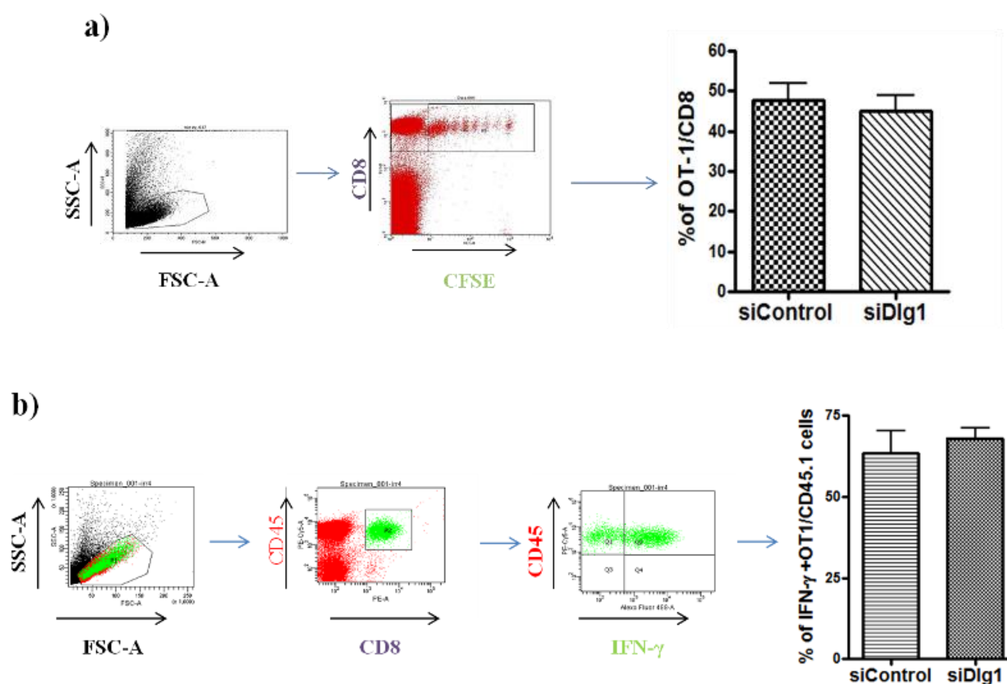

Figure 27. CD8+ T cell priming induced by wtDCs or Dlg1-depleted DCs in LNs. a) Mice were adoptively transferred with CD45.1 CFSE-labeled OT-1 cells. T cell priming was induced by injection of $3x10^5$ of Dlg1 depleted (or Control DCs) DCs, loaded with 0.5nM of MHC class I OVA peptide. Draining lymph nodes were harvested at day 3 post immunization to determine by FACS: **a)** the percentage of OT-1 cells over the total endogenous CD8 population. **b)** the ability of OT-1 cells to produce IFN-γ, following *ex vivo* restimulation and intracellular staining. Representative histogram plots demonstrate how injected T cells were screened for the priming capacity or for IFN-γ production. The values are represented as means ± SEM of two experiments with 5 mice each.

Discussion

Although macrophages and B cells have the capacity to present antigens to T cells and thus induce activation of adaptive immune response, dendritic cells (DCs) are, by many criteria, the most efficient APC of the immune system (Itano and Jenkins, 2003). DCs actually undergo an intrinsically imprinted program of maturation in order to acquire functional and phenotypical characteristics necessary for antigen presentation (Benvenuti et al., 2004b). Among many characteristics that endow these cells with the capacity to efficiently present antigens (Itano and Jenkins, 2003), one certainly important is that DCs directly contribute to the formation of informational synapse by actively rearranging their actin cytoskeleton. Specifically, Al-Alwan *et al*, have observed that DCs polarize fascin and F-actin toward synapse with T cells. This polarization of actin network was shown to be essential for T cell clustering and activation, as the number of synapses and T cells proliferative capacity were substantially decreased when DCs were pretreated with cytohalasin D (a drug that inhibits actin polimerization) (Al-Alwan et al., 2001a). The program of actin rearrangement is strictly controlled by DCs *via* temporal activation of Rho GTPases (Rac1 and Rac2) and WASp-mediated actin nucleation (Benvenuti et al., 2004a; Pulecio et al., 2008). Furthermore, mature DCs upregulate (ICAM-1) and actively cluster (LAT and LFA-1) adhesion molecules at the zone of IS, a process essential for the formation of stable conjugates and proper T cells differentiation (Benvenuti et al., 2004b; Scholer et al., 2008). Finally, utilization of two-photon microscopy has enabled further insights into the dynamic of DC-T cell contacts *in vivo* (Bousso, 2008) and highlighted the importance of DC-mediated contact duration in the induction of immune response or tolerance (Bousso, 2008; Hugues et al., 2004). The main message originating from these studies is that DCs do not represent a mere platform for the passive transmission of the signal but, quite opposite, T cell activation and differentiation appear to be a direct consequence of dynamical changes occurring in DCs during synapse formation.

Simultaneously, it is becoming increasingly evident that spatio-temporal interplay between specific polarity determinants occurring within the cells is essential to ensure proper cells functioning. For instance, spatio-temporal interplay between three polarity protein complexes (Par, Crumbs and Scrib) ensures the formation of functionally essential apico-basal polarity in epithelial cells (Bilder et al., 2000; Nelson, 2009). Migratory capacity of cells is a consequence of the acquirement of specific morphology based on global cytoskeleton polarization and formation of transient polarity proteins modules (Etienne-Manneville, 2008). In immune system, transient clustering of various proteins and temporal activation of signaling cascades beneath IS region, is well shown to enable adequate signal transmission (Krummel and Macara, 2006). With DCs having such a prominent role in ensuring the

formation of functional synapses and thus adequate T cells response, we wondered how DCs utilize and organize polarity determinants in order to fulfill their functional prerogatives.

At present, within immune system, it is well established that polarization of microtubule organizing center (MTOC) toward synapse affects not only IS stability but also the fate of engaged T cells. Intensive studies of this phenomenon did not only elucidate the mechanisms of MTOC translocation but delineated its importance in a multitude of processes: the early work of Kupfer *et al*, demonstrating that MTOC polarization occurs in T and NK cells (Kupfer et al., 1983; Kupfer et al., 1987), *via* its role as a major scaffolding unit in CTL and NK cells bringing lytic granules toward the target cells (Chen et al., 2006; Stinchcombe et al., 2006); these intensive studies have finally implicated the phenomenon of MTOC polarization in a new concept of asymmetrical cells division (*ACD*) of T cells (Oliaro et al., 2010). On the other side of the synapse, however, within DCs, nothing is known regarding the phenomenon of MTOC polarization toward IS.

In the present study we reveal that DCs cluster IL-12-rich vesicles toward DC-T cell interface, in antigen specific manner. Formation of specific synapse is further reflected in early, IL-12 dependent events occurring on T cell side: STAT4 phosphorilation and IFN-γ production. Moreover, we demonstrate that MTOC is a scaffold necessary to bring IL-12 vesicles for polarized delivery within IS, as inhibition of MTOC reorientation substantially affected early STAT4 phosphorilation and IFN-γ production. At later time point, interfering with MTOC negatively impacted survival and differentiation of T cells. Finally, we elucidate the mechanisms essential for MTOC polarization as we show that this phenomenon is dependent on Cdc42 and WASp activation.

Interleukon-12 (IL-12) is a “signature” cytokine produced by mature DCs. During T cell priming, IL-12 provides a third signal that has a decisive role in determining the fate of CD4+ T cells, as it potentiates the development of Th1 dominated response and simultaneously inhibits the development of Th2 dominated response (Trinchieri, 2003). Furthermore, IL-12 is essential to ensure activation and proliferation of CD8+ T cells (Pearce and Shen, 2007; Trinchieri, 2003; Valenzuela et al., 2002). Our experiments have shown that IL-12 positive vesicles were found enriched at the DC-T cell interface of antigen-specific conjugates in close proximity to VAMP-7 positive vesicles. VAMP-7 is a SNARE found on late endocytic vesicles and regulates the fusion with plasma membrane (Braun et al., 2004). Polarized secretion of cytolitic granules in NK cells was shown to rely on VAMP7-dependent exocytic pathway (Marcet-Palacios et al., 2008). Moreover, high magnification of synapse membrane area revealed IL-12 rich vesicles actually crossing the synapse. Downstream signaling cascade activated

upon engagement of IL-12R on T cell side involves activation of a specific Janus kinase (JAK2) which further phosphorilates a transcription factor (STAT4). Target gene for this transcriptional factor is a gene for IFN-γ whose expression represents an early signal for the complete polarization of T cell response (Bacon et al., 1995b; Lund et al., 2004). Our experiments demonstrate that the formation of antigen-specific synapse on DCs side is indeed reflected in IL-12 dependent events occurring in T cell: enhanced phosphorilation of STAT4 as well as early production of IFN-γ protein.

The possibility of synapse being a preferential route of cytokine secretion has been documented before. In an extensive study employing live-cell imaging and surface-mediated secretion assay in order to follow trafficking and secretion of various cytokines in Th cells in synapse with APC, Huse *et.al.,* have clearly demonstrated IL-2 and IFN-γ are specifically secreted in synapse area (Huse et al., 2006). Synapse formation between NK and DCs triggers polarization and secretion of IL-12 by DCs toward bound NK (Borg et al., 2004). Recently, an array of reports that tackled the phenomenon of polarized secretion in the cells of the immune system has helped reveal the existence of very well organized network enabling polarized secretion of soluble molecules. For instance, the works of Stinchicombe *et al*. revealed that synapse itself is an extremely complex structure where adhesion, signaling and secretion domains are very well differentiated (Stinchcombe et al., 2001b). The possibility that postranscriptional modification of microtubules predestines them to become specific pathways of vesicular transport has also been suggested (Hammond et al., 2008). The involvement of the small Rab GTPases enabling an initial contact between different vesicles has also added up to the complexity and specificity of the vesicle transport (Griffiths et al., 2010). Finally, the involvement of a pool of SNAREs (*soluble NSF attachment protein receptor*), ensuring specificity of membrane fusion events has also been established (Stow et al., 2006).

Within TLR stimulated DCs, IL-12 was localized with Golgi complex in the area of MTOC at all time points post stimulation. As we detected that the peak of IL-12 production (6h) is synchronized with the highest DCs' MTOC reorientation, up regulation of co-stimulatory molecules and maximum ability to cluster and activate T cells, we focused our attention on the functional study of IL-12. Single-cell experiments demonstrated that indeed MTOC and IL-12 are enriched at DC-T cell interface, in antigen-dependent manner. IL-12 clustering at the synapse area was shown to be dependent on the presence of microtubule network as the treatment of DCs with colchicine prior to synapse formation significantly reduced the percent of IL-12 clustering at the synapse. We observed a marked decrease in the number of DCs with IL-12 clustered at the synapse, when MTOC translocation has been

abrogated (in Cdc42 silenced DCs). These experiments suggested that MTOC might be in charge for bringing IL-12-rich vesicles toward the synapse area.

Within the immune system, the functions ascribed to MTOC have been numerous and vary with the cellular context. In migrating T cells, MTOC reorientation toward uropod might have a role in establishing a preferential direction of secretion, most likely *via* post-transcriptional modifications of MTs (Hammond et al., 2008; Krummel and Macara, 2006). The role of MTOC as one of the main players ensuring asymmetrical cell division (ACD) of T cells, upon prolonged contact with APC, has been recently suggested (Oliaro et al., 2010). During the initial phase of synapse formation, however, MTOC reorientation has been invariably associated to the process of polarized secretion within IS. Specifically, numerous reports state the role of MTOC as a general scaffolding structure necessary to deliver lytic granules toward synapse both in CTL and NK cells (Banerjee et al., 2007; Chen et al., 2006; Stinchcombe et al., 2006). Our results clearly stated that MTOC reorientation toward the synapse in DCs is important to ensure IL-12-dependent events in T cells. Absence of MTOC polarization (Cdc42-depleted and colchicine) subsantially decreased IL-12 dependent events occurring very early upon synapse formation in T cells: STAT4 phosphorilation and IFNγ synthesis. At later time points following synapse formation, interfering with MTOC polarization did not affect proliferative capacity of T cells, but negatively impacted the survival of divided T cells. This is in line with the reports stating the role of IL-12 in inducing T cell activation and survival (Valenzuela et al., 2002) most likely by upregulating the expression of anti-apoptotic genes (Bcl-2 and Bcl-3) (Li et al., 2006). Finally, production of IFNγ at later time points was strongly decreased in T cells primed with Cdc42-depleted DCs, These results are consistent with the role of IL-12 as the third signal that promotes DC-T cell interaction time necessary to induce the transmission of information and differentiation of T cells (Bousso, 2008; Henry et al., 2008; Hugues et al., 2004; Scholer et al., 2008).

The possibility of cytokine clustering at the synapse in DCs has been previously suggested as two cytokines (IL-12 and IL-18) were found enriched in the area of DC-NK interface (Borg et al., 2004; Semino et al., 2005). Moreover, initial contact between CD8+ T cells and DCs induces secretion of IL-1β vesicles from DCs (Gardella et al., 2001). In this study we further extend these observations in the context of CD8+ naive T cells priming and provide a molecular mechanism to explain cytokine recruitment at IS *via* polarization of the microtubule cytoskeleton. By immunofluorescentlly tracking MTOC, we have observed that DCs polarize MTOC toward the contact region with a T cell in manner that was dependent on TLR stimulation and Ag recognition by T cells.

Moreover, we observed that the incidence of MTOC polarization was dependant on the dose of antigen used, indicating that TCR alone is not sufficient to induce MTOC polarization. This was further proven as MHC I coated beads failed to reconstitute MTOC reorientation (not shown).

Although there is an array of reports where the activation of T cells as the function of antigen dose presented by DCs was followed *in vitro* and *in vivo,* the exact signal that triggers the activation of T cells is still not identified (Bousso, 2008; Bousso and Robey, 2004; Henrickson et al., 2008). Specifically, by using two photon microscopy and confocal analysis, Bousso *et al*., were able to notice that CD8+T cells formed more stable interactions and underwent intense proliferation when primed with DCs expressing higher doses of antigens (1µM). On the contrary, low-avidity interactions (30pM) failed to induce T cell clustering and lowered the level of T cell proliferative cycles. Following activation of T cells primed by DCs expressing different doses of peptide, Henrickson *et al.,* set up a threshold number of MHC-Ag complexes (ca.100) minimum to induce T cell activation. Even more, the capacity of T cells to proliferate and differentiate was a direct consequence of the number of DCs presenting antigen (Henrickson et al., 2008). Although these studies imply that activation of T cells is directly proportional to the dose of antigen presented by DCs or to the time of interaction (Mempel et al., 2004), the fact that DCs and T cells were shown to form both transient and stable interactions in the presence of peptide (Bousso, 2008) suggests that some other molecules are also involved in signaling *i.e.* T cell activation.

One possible scenario is that initial TCR recognition induces affinity changes of integrins that in turn further amplify the signal. This is in line with the observations that DCs upregualte ICAM-1 following TCR signaling and that ICAM-1 deficient DCs (in the presence of peptide) established only short interactions with T cells and failed to induce IFN-γ production in T cells (Scholer et al., 2008). Furthermore, the engagement of LFA on DCs side further stabilizes the synapse as it enhances MHC expression (de la Fuente et al., 2005) and was shown to induce clustering of two kinases (focal adhesion kinase and prolin rich tyrosine kinase) whose role in microtubular dynamics has been well established (Rodriguez-Fernandez et al., 2010). Direct evidence that LFA-1 signaling is a determining step in T cell activation and proliferation was also shown (Balkow et al., 2010). Finally, the role of integrin-mediate polarization events is not surprising as their involvement has been shown in other models system as well. For instance, in migrating astrocytes, initial integrin-mediated clustering of Par6-aPKCζ induces (*via* Dlg and APC clustering) MTOC repositioning toward the leading edge (Etienne-Manneville and Hall, 2001; Etienne-Manneville et al., 2005). As a consideration for the future experiments, using

ICAM-1 deficient DCs might be a way to assess the contribution of integrins in the phenomenon of MTOC polarization.

The level of MTOC polarization toward antigen-specific synapse in DCs is a function of DCs maturation level, as we have seen that this event increases with time post TLR engagement to be reaching its peak at 6h. It has been well documented that DCs acquire many characteristics during maturation that bestow them with the capacity to form stable conjugates with T cells and induce stronger signaling (Benvenuti et al., 2004b; Hugues et al., 2004). DCs ability to reorient MTOC toward a T cell could be one of the characteristics that DCs gain through maturation program, likely to be associated with better antigen-presenting capacity. In addition, intrinsic remodeling of actin cytoskeleton induced by TLR engagement (West et al., 2004) might in turn be responsible for microtubules rearrangement; actin and microtubules were shown to respond simultaneously to the same signaling and were found physically and functionally associated underneath the synapse area in T cells (Banerjee et al., 2007; Kodama et al., 2004).

By applying time-lapse video microscopy we were able to follow the dynamic of MTOC movement in DCs in time. We have noticed that, once polarized (7min post synapse formation), MTOC remains localized beneath synapse region, with the small oscillation on the spot, for the whole time of the movie (not shown). Constant presence of MTOC in synapse region could be suggestive of its direct role in processes occurring within IS region. The oscillations underneath the synapse membrane have also been noticed to occur in cytotoxic T lymphocytes during target mediated killing (Kuhn and Poenie, 2002). As microtubules represent the major pathway employed by endo/exocytic machiner, the small oscillations may reflect the dynamic of constant vesicle release and uptake occurring in the synapse region (Das et al., 2004; Griffiths et al., 2010).

MTOC polarization is a highly organized event shown to involve numerous proteins and signaling cascades. In our study we have revealed two regulators ensiring MTOC reorientation toward the synapse: Cdc42 and WASp. Cell division cycle protein (Cdc42) is a small Rho GTPase shown to be regulating numerous polarity processes in various cell types, by directly or indirectly affecting cytoskeletal reorganization (Etienne-Manneville, 2004). In the immune system, Cdc42 has been directly involved in MTOC polarization toward the place of phagosome formation in macrophages (Eng et al., 2007) and toward the synapse in NK and T cells (Chen et al., 2006; Stinchcombe et al., 2006). DCs in which Cdc42 has been silenced were characterized with morphological abnormalities as they had a more flat and spread appearance, lacking membrane protrusions. This is in line with the report where the

absence of Cdc42 compromised DCs migrational capacity as it induced defects in temporal and spatial coordination of leading edges, which further reflected in complete absence of coordination and loss of directional movement (Lammermann et al., 2009). The exact mechanism implicated in MTOC reorientation toward the synapse might involve various proteins and signaling cascades shown to be downstream effectors of Cdc42 (Blanchard et al., 2002; Dustin and Cooper, 2000; Stinchcombe et al., 2006).

Wiskott - Aldrich syndrome protein (WASp) has always been regarded as one of the main regulators of actin polimerization (*via* Arp2/3 complex) in various cell types and contexts (Blundell et al., 2010). However, a few fairly recent studies link microtubular and actin cytoskeleton and suggest WASp as one of the main factors directly responsible for MTOC reorientation underneath the synapse in NK cells (Banerjee et al., 2007; Kodama et al., 2004). Confocal analysis assessing the relative position of MTOC in wtDCs and WASp-/- DCs, in relation to antigen-specific synapse with T cells, revealed that WASp deficient DCs indeed demonstrate a striking defect in MTOC reorientation. However, further experiments aimed to understand the levels of IL-12 in WASp deficient DCs upon TLR signaling revealed that WASp deficient DCs produced much less IL-12 than wtDCs. It has been well established that WASp depletion in DCs causes numerous defects on DCs functioning and morphology due to a complete deregulation of actin cytoskeleton polimerization: loss of migrational capacity *in vivo* and *in vitro*; the inability of DCs to make stable contact and activate T cells *in vivo* and *in vitro* (Bouma et al., 2011; de Noronha et al., 2005; Pulecio et al., 2008). Our results further deepened the role of WASp in the proper functioning of DCs as they implied that WASp deficient DCs had an intrinsic defect in IL-12 production. To our knowledge this is the first result of WASp suggested involvement in cytokine production and it would be of great value understanding the mechanisms and signaling pathways that link TLR signaling with abrogated IL-12 production in WASp-deficient mice. Although WASp deficiency in DCs opened new avenues for research and it had a strong effect on the level of MTOC translocation, the intrinsic defected in cytokine production of WASp deficient DCs hampered its role as a putative model to test the functional effects of MTOC translocation in T cells.

Overall, our results strongly demonstrate that MTOC represent a scaffold structure necessary to bring the exocytic machinery (Golgi and IL-12 vesicles) toward the synapse region. IL-12, a cytokine ensuring the proper fate determination of T cells (Pearce and Shen, 2007; Trinchieri, 2003; Valenzuela et al., 2002), expresses its effects by being secreted preferentially (not exclusively) in the synapse area. This is in line with many reports stating the existence of preferential route of secretion of

different cytokines as a function of their role (Huse et al., 2006; Stinchcombe and Griffiths, 2003). Furthermore, DCs capacity to polarize MTOC and enable polarized IL-12 secretion adds up to the list of characteristics acquired by DCs upon their maturation and strengthens the position of mature DCs being best APC of the immune system. Finally, a well established idea that the decision regarding the differentiation pathway toward a specific lineage occurs at later time points has been well shaken by the data demonstrating that this decision occurs very early upon synapse formation (Celli et al., 2008; Clark et al., 2011). As STAT4 phosphorilation was strongly visible only 30 min upon synapse formation while the presence of its downstream target (IFN-γ) was clearly evident 2h upon synapse in T cells, our data support and further develop the notion that the decision toward the specific lineages occurs early upon synapse formation.

Enormous scientific effort in the field of polarity has helped elucidate the sophistication and importance of polarization processes in various cells and contexts; and DCs do not seem to be an exception (Bloom et al., 2008; Eun et al., 2006). Having established that antigen-presenting capacity of DCs has its roots in the adequate temporal and spatial orchestration of polarization events (MTOC reorientation and synapse-specific release of IL-12), we went on to investigate other possibly players enabling the establishment of polarized state in DCs. The presence (role) of evolutionary conserved polarity proteins belonging to three complexes (Scrib, Par and Crumb) has been shown irreplaceable for the acquisition of polarized phenotype in various cells and aspects *e.g.* migratory capacity, asymmetrical cell division in embryonic and non embryonic tissues, domain-specific functions of epithelial cells, secretion, signal transmission in neurological synapse. Intensive research in the field of polarity within the cells of the immune system has revealed that redistribution of polarity proteins is an imprinted property of all leukocytes. For instance, T cells and neutrophilles preferentially cluster Scrib, aPKC and Par6 within different domains, a process essential for their locomotion (Ludford-Menting et al., 2005; Real et al., 2007). Par1 clustering beneath the synapse region by T cells is essential for proper synapse maintenance as it further induces MTOC reorientation (Lin et al., 2009). Finally, Scrib/Dlg and Par3/aPKC are unevenly split within progeny cells arising from asymmetrical cell division (ACD) in a T cell (Oliaro et al., 2010), which is a likely cause of different fates of these cells (Chang et al., 2007). Another aspect that stimulated such an intense scientific interest in the function polarity proteins may have in the cells of the immune system comes from the realization that spatio-temporal deregulation of polarity proteins pathway is a direct cause of leukemogensis (Hawkins and Russell, 2008). Even though the contribution of polarity complexes to the establishment of functionally important phenotype has

been described in various cells in immune system, the question of what role they might have in DCs is still unanswered. Preliminary studies aiming to depict the polarization game occurring in DCs have reported that DCs actively cluster sphinophilin (a PDZ containing protein) and plexin-A1 underneath the synapse region with T cells, which is further important for the activation of the immune response (Bloom et al., 2008; Eun et al., 2006). Given the importance DCs have in adequately priming the activation of adaptive immune system and the emergence of polarity processes as the basis of adequate function acquirement, we reasoned it would be of great importance understanding how DCs utilizes polarity proteins in order to perform their functions.

In our work we revealed a preferential place of Dlg1 localization within immature and mature DCs as well as its post-transcriptional modifications occuring as the result of TLR stimulation. Furthermore, initial functional experiments imply that Dlg1 does not have a role in directional migration of DCs whereas its alignement at DC-T cell interface effects early IFN-γ production in T cells *in vitro.* At later time points, Dlg1 contributes to the establishment of a polarized T cell response.

In mammalian system, Dlg is presented in four isoformes Dlg1-Dlg4. Although they have all been shown to regulate numerous processes in most aspect of cell functioning, they appear to have a different distribution within tissues (Humbert et al., 2003; Humbert et al., 2008). Dlg1 appears to have a more diffused distribution, its role in establishing polarity has been well documented in various contexts and it is the closest homologue to *D.melanogaster* dmDlg since its transfection in fly mutants can functionally replace the absent Dlg protein and establish epithelial cell polarity (Humbert et al., 2008; Thomas et al., 1997). Most importantly, the role of Dlg1 in enabling various polarization aspects of T cell functioning has been undoubtedly demonstrated. For instance, Dlg1 polarizes toward the synapse in T cells, where it functionally associates to TCR and induces the activation of gene expression *via* p38 kinase activation (Rebeaud et al., 2007; Rincon and Davis, 2007); Dlgh1 polarization toward synapse post TCR activation was shown to enhance TCR clustering, autocrine cytokine production (IL-2 and IFN-γ) and enhanced NFAT signaling (Round et al., 2005). Finally, Dlg has been demonstrated to affect MTOC translocation toward IS in T cells, most likely through Dlg-dependent NFAT activation (Lasserre et al., 2010).

Important feature of DCs is their ability to migrate, upon TLR engagement by pathogen. This characteristic is essential for the proper activation of adaptive immunity as it enables DCs to bring pathogen derived sequences for T cell recognition within IS. The acquisition of migrational phenotype is characterized by the formation of actin-based protrusions (lamelopodia, filopodia), MTOC reorientation

toward one pole of the cell and asymmetrical distribution of polarity protein complexes. Formation of these transient polarity protein modules has a role in enabling adequate migration of various cells (Etienne-Manneville, 2008). In migrating T cells, however, the role of polarity proteins has just started to be revealed. Thus, T cell migration is enabled by Par3 and PKCζ clustering at the leading edge in response to chemokine signals (Gerard et al., 2007). Dlg and Scrib are specifically located at the region formed at the rear end of the migrating T cells (uropod). Loss-of function experiments have demonstrated that Scrib localization within uropod is essential for T cell migration (Ludford-Menting et al., 2005). The role of Crb complex in migratory capacity of the cells of the immune system, as well as others, is still very poorly understood (Etienne-Manneville, 2008; Krummel and Macara, 2006). By fluorescentlly tracking Dlg1 in DCs at various times post TLR stimulation we revealed that this protein abandons its localization underneath the plasma membrane and clusters in newly created membrane protrusions. Morphologically, these protrusions resemble the slender membrane structure forming at the rear end of the cell, called uropod, essential to transiently “lock” the cell to substratum (Krummel and Macara, 2006)

Recent study from Maneville *et al.,* suggest direct involvement of Dlg in the establishment of cellular migratory capacity. Specifically, Dlg1 clustering at front of the cell was shown to induce MTOC polarization toward the leading edge of the migrating astrocytes *via* its binding to GKAP (a scaffolding protein) and dynein (Manneville et al., 2010). In the cells of the immune system, role of Dlg in the induction of migration is still rather descriptive as it has been invariably observed at the uropod of the cells (Etienne-Manneville et al., 2005); however, its direct contribution to the migrational capacity of the cells of the immune system has not been tackled so far. Our data demonstrate that Dlg1 does not have a role in migratory capacity of DCs as Dlg1-deficient DCs did not show a defect in migratory capacity *in vitro* and *in vivo.* In motile astrocytes, Dlg has been invariably shown to cluster at the leading edge where it has numerous and important functions: it is directly involved in the recruitment of GKAP toward the leading edge; it is important for the association between dynein and microtubules; it regulates membrane attachment of plus-end of microtubules *via* association APC at the leading edge. All these functions eventually induce centrosome polarization toward the leading edge. However, although Dlg depletion completely inhibits MTOC reorientation and induces complete loss of cellular orientation, it does not affect the formation of the membranous protrusions (Etienne-Manneville, 2008; Etienne-Manneville et al., 2005; Manneville et al., 2010). Therefore, we reasoned that Dlg1 absence *per se* did not interfere with the capacity of mature DCs to migrate but it might have affected

their capacity to rieorent MTOC. MTOC is an important player in cellular migration mostly as it ensures the directionallity of the movement; however a few fairly recent reports state that MTOC position is not unchangeable within neuronal cells and argue its involvement in actually "leading" the migration (Distel et al., 2010; Umeshima et al., 2007). This is in line with many reports emphasizing the notion that the orchestration of intracellualr componentes essential for migration varies with specific cell type, context and environmental cues (Dow and Humbert, 2007; Etienne-Manneville, 2008; Humbert et al., 2006). Future experiments will address the questions whether Dlg1-depletion might affect MTOC localization in motile DCs and how this phenomenon is related to DCs migratory capacity.

Our previous results regarding MTOC and IL-12 polarization, as well as increasing number of reports imply that DCs'dynamical changes and polarization events occuring during DC-T cell crosstalk are an absolute prerequisite for the proper activation of adaptive immunity (Bousso, 2008; Hugues et al., 2004). An ever increasing body of literature, as earlier mentioned, regarding Dlg localization in T cells, undoubtedly states that Dlg is clustered within IS in these cells. Dlg function in synapse is still, however, a matter of controversy, as it was shown that Dlg can either activate (Lasserre et al., 2010; Round et al., 2005) or negatively regulate synapse formation and signaling (Stephenson et al., 2007; Xavier et al., 2004). Our experiments clearly indicated a fast (5min) shift of Dlg1 in DCs toward antigen-specific synapse with T cells, a pattern that further strengthened with time (30min). Interfering with Dlg1 had shown a strong decrease in the events occuring very early upon synapse formation. Specifically, as early as 1h post synapse formation, we have observed lower levels of IFN-γ mRNA in T cells engaged in antigen-specific synapse with Dlg1-depleted DCs. Furthermore, Dlg1-depletion in DCs did not have an effect on initial activation of T cells wheras, at later time points it strongly decreased T cell capacity to produce IFN-γ *in vitro*. These results are in accordance with the *in vivo* observations that early activation of T cells (CD69 upregulation) occuring during the initial contact between DC and T cell happens even in the absence of specific synapse; however a formation of strong, specific and long-lasting contact is necessary for the proper differentiation of T cells at later time points (Bousso, 2008; Hugues et al., 2004; Mempel et al., 2004). Collectively, these results imply that, during synapse formation, Dlg1 in DCs is necessary to ensure an initial "boost" toward IFN-γ –dominanted T cell pathway that is later reflected in the establishment of a higly polarized T cells response.

Important to say, however, is that proliferative and IFN-γ producing capacities of T cells were not compromised when T cells were primed with Dlg1-depleted DCs *in vivo.* There may be a few possibilities for the discrepancy between the data regarding T cell activation by Dlg1 depleted DCs *in*

vitro and *in vivo.* Since transfection procedure itself affects DCs vitality, we reasoned that the professional phagocytes (DCs and macrophages) in recipient mice may take up and process the proportion of transfected cells that are dying and present OVA peptide themselves; thus abolishing potential difference in antigen presentation by Control *vs.* Dlg1-depleted DCs. Another possibility is that the high peptide dose used to induce priming by transfected DCs may mask the putative difference in priming capacity of Dlg1 depleted DCs *vs.*Control DCs.

Confocal analysis at single cell level indicated that Dlg1 depletion compromises the capacity of DCs to reorient MTOC toward synapse, in antigen-specific manner. These results are in accordance with a recent study performed by Lasserre *et al.* where they noticed that in T cells, MTOC translocation is Dlg dependent and state that this occurs most likely *via* p38/NFAT dependent pathway (Lasserre et al., 2010). A link between microtubular cytoskeleton and Dlg has been reported even before in T cells as Hanada *et al.,* suggested synchronized movement of Dlg and MTOC toward a lymphocyte cap (after CD2 binding) *via* a kinesin motor GAKIN (Hanada et al., 2000). As it is very well documented that IFNγ production in T cells is a direct consequence of IL-12 binding to its receptors (Lund et al., 2004) and we have previously established that MTOC is a scafold necessary to bring IL-12 rich vesicles for the polarized secretion within IS; it is tempting to speculate that Dlg1 is implicated in the process of polarized cytokine delivery by direct regulation of MTOC movement. Although our preliminary results imply that Dlg1 and MTOC follow the same kinetics of distribution toward IS, further experiments are needed in order to dechiper functional and causal relationship between the phenomenon of MTOC and Dlg1 reorientation.

Consistent with the acquisition of migrational and antigen-presenting capacity, DCs undergo a complete turnover in their genetical program. Specifically, DCs lose the capacity to recognize and engulf pathogens due to a decrease in receptor expression and downregulation of phagocytic capacity. Furthermore, the expression of specific chemokine receptors (CCR7) is augmented enabling DCs to respond to chemokines (CCL19 and CCL21), produced by stromal cells in lymph nodes. Production of specific set of cytokines (IL-12 and TNF-α) and upregulation of co-stimulatory molecules (CD80, CD86 and CD40) completes the maturation program, rendering DCs capable to present antigens to T cells (Banchereau and Steinman, 1998; Sallusto et al., 1998). We have checked whether DCs maturation program might include the changes in Dlg1 gene expression levels and/or post-transcriptional modifications.

Real-time RT-PCR and Western blot analysis of DCs stimulated with TLR agonists for various times, clearly demonstrated that DCs do not change the rate of Dlg1 expression as a result of maturation. This might be due to the fact that polarity proteins are constantly necessary to enable the formation of transient polarity modules in various functions of DCs. The maintenance of a definitive pool of polarity proteins within various cells is a prerequisite for adequate polarity-determined processes, as the changes in their expression are intimately associated with disruptions of cell polarity and, in most drastic cases, tumorogenesis (Humbert et al., 2003; Humbert et al., 2008). In accordance with that is the fact that oncogenic forms of human papiloma viruses (HPV16 and HPV18) have explicitly evolved the mechanism to induce a polarity imbalance (by degrading only a certain portion of Dlg and Scrib) as the means of their survival and proliferation (Thomas et al., 2008).

Phosphorilation has been shown to be a main post-transcriptional modification of Dlg in various cell types and contexts (Thomas et al., 2008). The effects of phosphorilation of Dlg include change in its location, change in the activation status or even change in its function (Mantovani and Banks, 2003; Massimi et al., 2006; Sabio et al., 2005). Within the immune system the data regarding the change in Dlg phosphorilation status have been observed in T cell, as a result of specific signals arising from TCR activation. Specifically, by employing immunoprecipitation techniques Hanada *et al.,* have reported that Dlgh1 and Lck interact in Jurkat T cells; while Xavier *et al.* further dissected their functional relationship reporting that a certain pool of Dlgh1 undergoes Lck-dependent phosphorilation as the result of TCR signaling (Hanada et al., 1997; Xavier et al., 2004). Physical and functional association between Dlg and p38 (a mitogen activated protein kinase), as the result of TCR signaling was also observed (Adachi and Davis, 2011; Round et al., 2005). Here we demonstrate that a certain proportion of phosphorilated form of Dlg1 is present in the steady state conditions (immature DCs). However, TLR stimulation of DCs induces striking changes in phosphorilation status, as the whole non-phosphorilated fraction of Dlg1 present in the cells undergoes hyper-phosphorilation as early as 30min post TLR stimulation. Cells appear to be maintaining functional equilibrium by constantly shifting between phosphorilated and nonphosphorilated forms of various proteins (Balsamo et al., 1996; Yap et al., 1997). Further data supporting this idea are coming from the work of Koh *et al.* who demonstrated that Dlg is held at the synapse borders in T cell *via* constant balance between the rate of phosphorilation by CaMKII kinase. Specifically, these authors observed that either inhibiting CaMKII or using constitutively active mutant induce Dlg mislocalization (Koh et al., 1999). Thus, the presence of phosphorilated form of proteins within immature DCs might reflect the necessity of certain pool of

phosphorilated Dlg1 for the maintenance of basal level of polarity processes. However, as TLR engagement induces drastic changes in cell's morphology it could be speculated that all of Dlg1 is necessary in order to maintain DCs morphological prerogatives. This result suggests a novel link between TLR stimulation and changes in post-transcriptional modifications. This was not further pursued in this work but it would be of great interest to understand the pathways linking these processes.

In immature DCs, we observed that Dlg1 is expressed at much higher levels than in T cells. The general idea that DCs, rather than T cells, are the ones that govern the dynamic of the immune response has already been implied before. Already in 2001, Al-Alwan et al., have seen that, during synapse formation, inhibiting actin polimerization within DCs affects T cells activation more dramatically than the corresponding process in T cells (Al-Alwan et al., 2003). Actual *in vivo* visualization of processes occurring within the synapse has implied that it is the dynamical changes of DCs membranous protrusions the thing that ascertains transmission of the signal and stability of the synapse (Bousso, 2008; Hugues et al., 2004). With Dlg1 being such a prominent and multifunctional protein in polarity processes, its higher expression might be a reflection of its importance in DCs functional dynamism. Immunofluorescent tracking of Dlg1 localization in immature DCs implied that it is mainly expressed within actin clustered networks at cell's periphery. Dlg stabilization in the membrane area is consistent with them being MAGUKs (*membrane associated guanilat kinases*) containing PDZ-domains which enable them to bind various conserved motifs present at the C-terminus of many membrane molecules (Harris and Lim, 2001). In T cells, Dlg is physical "locked" at cell periphery by ezrin-radixin-moesin (*ERM*) complex, prior to their activation (Ludford-Menting et al., 2005; Lue et al., 1996). ERMs contain two domains enabling this complex to simultaneously bind to transmembrane molecules (*e.g.* ICAM-1, CD43, CD44) and actin cytoskeleton (Louvet-Vallee, 2000). Synapse formation represents the activation signal for ezrin (Faure et al., 2004), as it induces its phosphorilation-dependent conformational relaxation and clustering toward the synapse. Ezrin movement toward synapse is accompanied by Dlg movement and MTOC translocation (Lasserre et al., 2010). The importance of Dlg at the synapse was demonstrated by the defects in the synapse topology, perturbation of cellular morphology and absence of MTOC translocation in T cells when Dlg was silenced. Hence, homologues to the situation occurring in T cells, it could be hypothesized that Dlg1 is localized at cellular periphery in DCs "waiting" for the activation signals emerging from the synapse upon which it would cluster in the synapse area.

The specific signal that induces Dlg1 clustering toward the synapse, however, has not yet been found. Specifically, we have noticed a certain level of Dlg1 enriched beneath the contact region with T cell, in unspecific manner. This most likely suggests that the initial clustering of Dlg1 is not initiated by antigen recognition, but it is however further strengthened by the addition of antigen. In migrating astrocytes, for instance, it has been demonstrated that integrin-mediated clustering of Cdc42 at the leading edge induces further recruitment of Par6-aPKCζ (Etienne-Manneville and Hall, 2001) as well as Dlg and APC (Etienne-Manneville et al., 2005). Thus, one likely hypothesis might be that initial clustering of Dlg1 toward the synapse region is mediate by integrins and that TCR activation induces further affinity changes of integrins, amplification of the signal and stronger Dlg1 clustering. This is in line with the reports stating that the polarization events are the outcome of transient signaling complexes being activated as the result of specific composition of proteins present (Aranda et al., 2008; Hawkins and Russell, 2008).

Conclusions

The notion that DCs are a mere platform ensuring passive transmission of the signal is an archaism. Enormous scientific effort has revealed that DCs actively contribute to the formation of informational synapse by clustering actin cytoskeleton, upregulating and clustering adhesion and signaling molecules within the IS and by temporally regulating specific signaling pathways. The emergence of two-photon microscopy, enabling actual visualization of the processes occurring between DCs and T cells in lymph nodes of live animals, has revolutionized the field of immune interactions as it emphasized that DCs-mediated contacts directly shape T cells fate.

Our work further highlighted this notion as we revealed several aspects acquired through DCs maturation program that directly influence T cell destiny. We demonstrated that DCs preferentially secret cytokines (IL-12) within the synapse area. Apart from being secreted in such a manner in order to avoid harmful effects on bystander cells, polarized secretion of IL-12 is integrated exclusively by target T cells as a specific signal that promotes their survival and differentiation. Specifically, polarized IL-12 secretion induces early STAT4 phosphorilation and IFN-γ production in naïve CD8+T cells; whereas, at later time-points, it enhances the survival of activated T cells and contributes to the complete polarization of T cell response. Furthermore, our data revealed the outskirts of, what is most likely, a highly organized pathway of polarized IL-12 secretion as this phenomenon was dependent on MTOC reorientation toward the synapse. The process of MTOC reorientation is highly regulated in DCs and depends on the activation of small Rho GTPase (Cdc42) and WASp. Within immature DCs, Dlg1 is mainly localized at cellular periphery whereas TLR stimulation induces Dlg1 relocalization and hyper-phosphorilation. During synapse formation, DCs actively cluster Dlg1 toward the synapse area; a process necessary to signal an initial “boost” toward IFN-γ–dominated T cell pathway that is later reflected in further polarization of T cell response.

Collectively, we conclude that MTOC-dependent polarized secretion of IL-12 and Dlg1 clustering within IS are characteristics acquired by DCs through their maturation program and are essential to ensure adequate polarization of T cell response. Contrary to long time accepted paradigm that T cell decision regarding differentiation pathway happens at later time points following synapse formation, our data support and further deepen the emerging notion that T cell fate is decided very early upon initial DC-T cell contact.

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