Identification of vesicular SNARE proteins involved in the secretory intracellular trafficking of IL-12 in dendritic cells

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Identification of vesicular SNARE proteins involved in the secretory intracellular trafficking of IL-12 in dendritic cells

Settore scientifico-disciplinare: Biologia molecolare

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To the people
who are my family,
in every conceivable way
ABSTRACT

Dendritic cells (DCs) are professional antigen presenting cells. They have the unique ability of recognizing antigens, internalizing and processing them for presentation on MHC complexes. DCs also express pattern-recognition receptors like Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns and induce DCs maturation. As a consequence DCs increase their antigens presenting ability, their levels of co-stimulatory molecules on the cell surface, and their production of cytokines, which are critical for inducing specific T cell activation. All these functions make DCs key elements in the immunological response against pathogens, viruses and tumors.

One of the most important cytokines in T cell priming, interleukine-12 (IL-12), begins to be largely transcribed immediately after TLR activation. We have previously shown that in DCs engaged in antigen specific immunological synapses (IS), IL-12 is found enriched in the proximity of the contact site between DC and T cells, and several evidences suggest that its secretion is polarized towards the T cell. These data indicate that polarized trafficking of cytokines containing vesicles in DCs is a mechanism to optimize T cell priming.

Polarized trafficking of soluble mediators through defined subcellular areas has been described as fundamental for the function of several other immune cells, but at present a clear characterization of the pathways and organelles implicated in the regulation of cytokine secretion in dendritic cells is still missing.

The family of SNARE (soluble-N ethylmaleimide sensitive-factor accessory-protein (SNAP) receptor) proteins, which regulate the membrane fusion process during the intracellular trafficking of soluble mediators, has emerged as the most informative for the identification of such pathways. Indeed different SNAREs regulate diverse mechanisms such as the constitutive secretion of immune mediators, phagocytosis and endocytosis, or also the release of inflammatory mediators and products from secretory granules.

During my thesis I have investigated the role of SNAREs in IL-12 secretion by DCs. I have analyzed the expression and modulation during maturation of three SNAREs, Syntaxin6, VAMP3 and VAMP7, that were previously shown to play a role in other immune cells. The subcellular localization and co-localization with the IL-12 subunits were analyzed in DCs alone and in the context of IS by immunofluorescence. I performed functional analyses by silencing
SNAREs with the siRNA strategy to detect impairment in DC cytokines secretion and in T cell priming. These studies led to the identification of the lysosomal-late endosomal VAMP7 as a specific regulator in the polarized secretion of IL-12 during T cell priming. This suggest a role, in the secretion of soluble newly synthesized mediators, of late endosomal organelles, until now thought to be implicated only in the regulated secretion of pre-constituted mediators such as cytotoxic granules. Furthermore VAMP3 was found to downregulate the production of all the cytokines tested; we propose this SNARE as regulator of the TLR9 signaling pathway.

The second part of the work stems from the concept that DCs properties become suppressed by the tumor microenvironment and many evidences suggest that tolerogenic DCs may be a crucial element in the development of tumor-mediated immune anergy. Different steps in the antigen presentation process are impaired, but also the pattern of secreted cytokines is skewed. In particular, how secretion of inflammatory cytokines becomes impaired in tumor-exposed DCs has not been investigated.

I set up a model of mice bearing Lewis lung carcinoma (3LL) to analyze DCs *in vitro* (co-culture) and *in vivo* (spleen and tumor-bearing lungs). In all the models I observed a strong impairment in IL-12 production, upon restimulation with TLR agonists. In spleen DCs subtypes remained unchanged during tumor progression, whereas in tumor-bearing lungs the DC composition varied, with an expansion of CD11b<sup>hi</sup> DCs and a reduction in CD103<sup>+</sup> DCs. DCs were isolated from all the three models to analyze the expression of trafficking proteins, and I found that tumor-exposed DCs downregulate all the SNAREs and in particular VAMP3. Moreover Rab27a, a GTPase implicated in the regulation of the exocytosis of secretory granules and lysosome-related organelles is also downregulated at protein level. These preliminary observations suggest that regulation of trafficking proteins in DCs may represent a novel pathway targeted during immunosuppression.
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<tr>
<td>3LL-D122</td>
<td>Lewis Lung Carcinoma cell line</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-Presenting cells</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP Ribosylation Factor</td>
</tr>
<tr>
<td>ARG1</td>
<td>Type I arginase</td>
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<tr>
<td>Batf</td>
<td>Basic leucine zipper transcription factor ATF-like</td>
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<tr>
<td>BM-DC</td>
<td>Bone Marrow-derived DC</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
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<tr>
<td>CCR</td>
<td>C-C chemokine receptor type</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>cDC</td>
<td>conventional DC</td>
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<tr>
<td>COP</td>
<td>Coating Protein</td>
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<tr>
<td>CpG</td>
<td>Cytidine-phosphate-Guanosine</td>
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<tr>
<td>CpG-ODN</td>
<td>CpG oligodeoxynucleotides</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony Stimulating Factor 1</td>
</tr>
<tr>
<td>cSMAC</td>
<td>central SMAC</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>Dlg1</td>
<td>Disks large homolog 1</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double strand RNA</td>
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<tr>
<td>EE</td>
<td>Early Endosomes</td>
</tr>
<tr>
<td>EEA-1</td>
<td>Early Endosome Antigen 1</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell sorting</td>
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<tr>
<td>Flt3L</td>
<td>FMS-like tyrosine kinase 3 Ligand</td>
</tr>
<tr>
<td>GC</td>
<td>Golgi Complex</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide Exchange Factors</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>G-MDSC</td>
<td>Granulocyte-MDSC</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
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<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>Id2</td>
<td>Inhibitor of DNA-binding 2</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukine</td>
</tr>
<tr>
<td>iMC</td>
<td>immature Myeloid Cell</td>
</tr>
<tr>
<td>IMDC</td>
<td>Immature DC</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 Receptor-Associated Kinase</td>
</tr>
<tr>
<td>IRF8</td>
<td>Interferon Regulatory Factor 8</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological Synapse</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KDEL</td>
<td>Lys-Asp-Glu-Leu</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>Lamp-1</td>
<td>Lysosomal-associated membrane protein 1</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans Cell</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte Function-associated Antigen 1</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LRO</td>
<td>Lysosome-Related Organelles</td>
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<tr>
<td>MDSC</td>
<td>Myeloid-Derived Suppressor Cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>M-MDSC</td>
<td>Monocyte-MDSC</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule Organizing Centre</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target Of Rapamycin</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NSF</td>
<td>N ethylmaleimide sensitive-factor</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAF</td>
<td>RNA Polymerase II Associated Factor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated Molecular Pattern</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid DC</td>
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</tbody>
</table>
PKC  Protein Kinase C
PM   Plasma Membrane
PRR  Pattern Recognition Receptor
pSMAC peripheral SMAC
pSTAT phosphorylated STAT
RE   Recycling Endosomes
ROS  Reactive Oxigen Species
siRNA small interfering RNA
SMAC Supramolecular Activation Clusters
SNAP Soluble-N ethylmaleimide sensitive-factor accessory-protein
SNARE Soluble-N ethylmaleimide sensitive-factor Accessory-protein Receptor
ssRNA single strand RNA
STAT Signal Transducer and Activator of Transcription
Stx  Syntaxin
TAM  Tumour-Associated Macrophages
TAP  Transporters Associated with antigen Processing
T-bet T-box expressed in Tcells
TCR  T cell Receptor
TGF-β Transforming Growth Factor beta
TGN Trans-Golgi Network
Th   T helper
TIDC Tumour Infiltrated DC
Tip-DC Tumour-necrosis factor– and inducible nitric oxide synthase–producing inflammatory DC
TIR  Toll/IL-R
TLR  Toll-Like Receptor
TNF  Tumour Necrosis Factor
TRAF6 TNF Receptor Associated Factor 6
Treg  Regulatory T cells
VAMP Vesicle-Associated Membrane Protein
VEGF Vascular Endothelial Growth Factor
Vti-1B Vesicle transport through interaction with t-SNARE 1B
WASp Wiscott Aldrich Syndrome protein
1. Introduction
1.1. Dendritic cells: the sentinels of the immune response

Dendritic cells (DCs) have been described for the first time in 1973, as a new population of large cells observed in mouse peripheral lymphoid organs, characterized by “pseudopods of varying length, width, form, and number, resulting in a variety of cell shapes ranging from bipolar elongate cells to elaborate, stellate or dendritic ones” (Steinman and Cohn, 1973).

This heterogeneous population of hematopoietic cells belongs to the class of professional antigen-presenting cells (APC), together with the macrophages and the B cells.

1.1.1. DC subtypes

In terms of cell characterization, many subpopulations of DCs have been identified, but there are four main categories in which they can be divided: conventional DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells and monocyte-derived DCs.

- cDCs are a heterogeneous population of hematopoietic cells characterized by high phagocytic ability and specialized antigen-processing and presenting features. CDCs reside in all lymphoid, interface, and connective tissues. One of their key feature is their short half-life (approximately 3–5 days) and continuous replacement from bone marrow precursors, a process dependent on the cytokine Flt3L (Waskow et al., 2008). Small number of DC progenitors rapidly transit from bone marrow to spleen and lymph nodes via the blood as relatively immature progenitors, and after several rounds of cell division they differentiate into cDCs. Peripheral lymphoid organ DC homeostasis is therefore dependent on the rate of DC progenitor input from blood, cell division and cell death.

Once completely differentiated, cDCs encounter and capture antigens in peripheral tissues and migrate via afferent lymphatic vessels to the T cell zones of secondary lymphoid organs to initiate adaptive immune responses.

Tissue-resident cDCs subtypes

One of the biggest challenges to understand the molecular mechanisms that control DC function has been the lack of specific markers to define the DC lineage.

Indeed, the common definition of DCs is that of hematopoietic cells that on one hand don’t express hematopoietic lineage markers (Lin⁻) and on the other hand express high amounts of MHC class II (MHC II^{high}) and the integrin CD11c (CD11c⁺).
This definition is very poor and misleading, as MHCII\textsuperscript{high} is shared by B cells and activated macrophages, whereas CD11c\textsuperscript{+} is found expressed also by some macrophage population, activated T cells, B cells and NK cells.

Progress has been made in recent years in understanding the origin and the transcriptional program that controls DC differentiation in vivo. These results allowed the study of homogenous cell populations that derive from committed precursors along a distinct differentiation program shifting the focus from studies on phenotypically defined subsets to developmentally regulated subsets.

It has been shown that two DC populations, lymphoid tissue CD8α\textsuperscript{+}CD11b\textsuperscript{low} DCs and non-lymphoid tissue CD103\textsuperscript{+}CD11b\textsuperscript{low} DCs share a common origin, defined by the transcription factors Batf and IRF8 (Edelson et al., 2010; Hildner et al., 2008; Tailor et al., 2008), and by the inhibitor of DNA protein Id2 (Edelson et al., 2010; Hacker et al., 2003) (Figure 1.1.). These DCs lack the expression of macrophage markers, their differentiation and expansion is promoted by the mammalian target of rapamycin (mTOR) (Sathaliyawala et al., 2010), and for both their development and homeostasis are critically dependent (nonlymphoid tissue CD103\textsuperscript{+}CD11b\textsuperscript{low} DCs overall) on the cytokine fms-like thyrsoine kinase 3 ligand (Flt3L) and its receptor (Flt3) (Ginhoux et al., 2009).

The most important peculiarity shared by CD8α\textsuperscript{+}CD11b\textsuperscript{low} and CD103\textsuperscript{+}CD11b\textsuperscript{low} DCs is their functional pattern, which gives them their large relevance as lymphoid and nonlymphoid inducers of the adaptive immunity.

In fact, they are very potent producers of interleukine-12, a fundamental CD8\textsuperscript{+} T cell-activating cytokine discussed more in detail in chapter 1.3.
Associated to this function, they are also very potent in cross-presenting antigens to CD8⁺ T cells and in inducing their differentiation to CD8⁺ effector T cells (del Rio et al., 2007; GeurtsvanKessel et al., 2008; Heath and Carbone, 2009; Kim and Braciale, 2009; Shortman and Naik, 2007).

- pDCs circulate in blood and lymphoid tissues, and are peculiar overall for the strong capacity to secrete large amounts of type-I interferons (Colonna et al., 2002). Similarly to cDCs, pDCs derive from common bone marrow progenitors and require Flt3L for differentiation, although pDCs diverge from cDCs during maturation in the bone marrow (Figure 1.1.). Moreover, after activation, pDCs change dramatically their appearance, passing from a spherical shape to a dendritic morphology, an observation that formed the basis for their original designation as DC precursors (Naik et al., 2007; Onai et al., 2007).

- Langerhans cells (LC) are the sole DCs in the epidermis. They were considered as the proto-typical tissue DC on which the ‘DC paradigm’ was based (Banchereau and Steinman, 1998; Romani et al., 2010). Their location at a barrier surface provides them with early access to skin pathogens, commensal organisms as well as to epidermal self-antigens. LCs transport these antigens to regional lymph nodes where they present to naive T cells thereby initiating adaptive cutaneous immune responses.

LCs are identified by their marker Langerin (CD207) (Valladeau et al., 2000), even if discrimination is not sufficient anymore, after the discovery of dermal langerin⁺ DCs, that conserve the characteristics of cDCs (Poulin et al., 2007).

Unique features of LCs are their radioresistance and the fact that they develop independently of Flt3L, but dependently of CSF-1 (Xu et al., 1995).

- Monocyte-derived DCs are short-lived cells that originate from blood monocytes in response to inflammation or infection and differentiate into ‘Tip-DCs’ (tumour-necrosis factor– and inducible nitric oxide synthase–producing inflammatory DCs). Tip-DCs, which were initially so called because of their dendritic appearance and expression of CD11c, are thought to represent the main inflammatory cell type during infection (Serbina et al., 2003).

The induction of DC differentiation in vitro from peripheral monocytes by granulocyte-macrophage colony stimulating factor (GM-CSF) first suggested that monocytes may be an
important reservoir for DC development (Inaba et al., 1992). Like cDCs, GM-CSF-derived DCs upregulate their expression of CD11c and MHC class II and efficiently stimulate naive T cells.

1.1.2. DC maturation
DC maturation is a developmental program that starts with antigen internalization and process and continues in the presentation of this antigen on the cell surface until the acquisition of T cell activation competence.

This mechanism is a highly complex, time-ordered process involving changes at many levels including gene expression, intracellular transport, cytoskeletal activity, and localization within the host (Olex et al., 2010).

In the classic paradigm (Langerhans paradigm) immature DCs are present in the peripheral tissues, where they show high capacity of “sensing” exogenous antigens and endocytizing them, and low presentation capacities.

After the encounter with the antigen the DCs get matured, up-regulating histocompatibility and co-stimulatory molecules, and starting to produce inflammatory cytokines.

On the other hand they down-regulate their endocytic/phagocytic activity, by changing rates of membrane turnover, cytoskeleton, cell morphology and cell migration activity, and these changes are induced by up-regulation of chemokines, chemokine receptors and adhesion molecules.

In the mean time they also increase their degradative capacity associated with down-regulation of protease inhibitors (Delamarre et al., 2005; Garrett et al., 2000; Huang et al., 2001; Trombetta et al., 2003; Turley et al., 2010).

Finally, DC maturation is also a terminal differentiation process marked by shut down of the cell cycle followed by the eventual programmed death of the cell (Bertho et al., 2002; Kim et al., 2005; Rescigno et al., 1998).

1.1.3. Antigen presentation

Antigens are macromolecules able to induce immune responses in the host organism. They are constituted by proteins, polysaccarides, or a conjunction of them with lipids, and are divided into exogenous and endogenous, based on their origin.

The exogenous antigens are recognized and captured by the antigen-presenting cells, that act internalizing them into endosomes by endocytosis, degrading them with the fusion of the endosomes with the lysosomal compartments, and displaying the resulting short fragments at the
cell surface together with the class II molecules, forming the major histocompatibility complex-II (MHC-II).

The endogenous antigens (generated within the cell such as viral peptides of infected cells) are degraded into proteasomes of the APC, brought to the lumen of the ER by transporters associated with antigen processing (TAPs), and presented at the cell surface on a class I histocompatibility molecule, forming the MHC-I.

**Figure 1.2. Antigen-presentation pathways in dendritic cells**

A variant pathway of processing and presentation of antigens is the cross-presentation, exclusive ability of the dendritic cells. In the cross-presentation the APC internalize dead cells containing the non-self proteins (viral proteins of infected cells, cell debris, aberrant protein of cancer cells) into lysosomes for the degradation. Once the proteins are free, they are brought to the proteasomes for the degradation, and from here continue the class I pathway (Figure 1.2.). This mechanism is practically the only way by which immune system can recognize and fight viral infections or mutations that occur in parenchymal cells (Rock and Shen, 2005; Villadangos and Schnorrer, 2007).
1.2. A bridge between innate and adaptive immunity

Dendritic cells can be considered a point of contact between innate and adaptive immunity. They are part of the *innate immunity* because of their capacity of recognizing antigens and discriminating them between the three main categories: *microbial nonself*, *missing self* and *induced or altered self* (Medzhitov and Janeway, 2002).

- The *microbial nonself* discrimination is based on the ability to recognize conserved products of microbial metabolism that are unique to microorganisms and are not produced by the host.
- The *missing self* discrimination looks at “markers of normal self”, that are unique to the host and absent from microorganisms, and such recognition triggers various inhibitory pathways that block initiation of immune responses against self.
- The discrimination of the third type of antigens, the *induced or altered self*, is based on the detection of markers of abnormal self that are induced upon infection (in particular, viral infection) and cellular transformation. These markers trigger cell elimination by the immune system.

**Microbial nonself recognition**

This capacity is crucial for the host defence. It consists in the detection of conserved molecular patterns that are essential products of microbial physiology, and allow the immune system to distinguish self from microbial nonself. These invariant structures are referred to as pathogen-associated molecular patterns (PAMPs).

*Figure 1.3. DC maturation triggers expression of MHC molecules, costimulatory signals and soluble cytokines, three main signals for T cell priming*
PAMPs are unique to microbes and invariant among microorganisms of a given class and are recognized by receptors of the innate immune system called pattern recognition receptors (PRRs) as molecular signatures of infection, and this recognition leads to the induction of an immune response (Medzhitov and Janeway, 2002). Many kind of PRRs have evolved to recognize microbial antigens; secreted PRRs bind to microbial cells and tag them for the elimination either by the complement system or by phagocytosis.

**Toll Like Receptors**

One of the best characterized classes of PRRs that directly contribute to the inflammatory response to pathogens is the Toll-like receptor family (TLRs).

TLRs are transmembrane proteins that recognize molecular structures expressed by different groups of micro-organisms. TLR can be expressed on cell surfaces, mainly recognizing bacterial products (as for TLR 1, 2, 4 and 6), or on the membrane of endocytic vesicles and endosomes, specializing in recognition of viral nucleic acids (as for TLR 3, 7 and 9) (Iwasaki and Medzhitov, 2004).

Each TLR has precise ligand specificities, expression patterns and target genes that can be induced. **TLR4** is critical in the recognition of lipopolysaccharide (LPS), a component of the outer membrane of Gram’ bacteria, whereas **TLR2** is pivotal to the recognition of bacteria peptidoglycan and lipoproteins, but is also able to bind components from fungi and parasites. Other TLRs are involved in the recognition of microbial proteins such as flagellin by **TLR5** or apicomplexan profilins by **TLR11**. TLRs are also essential in the antiviral immune responses. By inducing type I IFNs, they are able to initiate the immunity towards viral infections. For instance, **TLR9** and **TLR7** are crucial in the recognition of unmethylated 2’-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs present in DNA viral genome, or ribonucleic acid homologs (imiquimod and resiquimod) and ssRNA rich in guanosine/uridine, respectively. A further member, **TLR3**, responds to dsRNA and to its synthetic analog polyinosinic acid-cytidylic acid (poly(I)•poly(C)) (Kawai and Akira, 2006).

Upon activation, TLRs can follow two distinct activation pathways, that involve adaptor proteins characterized by the expression of Toll/IL-R (TIR) domains. The most known and implicated in nearly all the TLR activation pathways is **MyD88**. This adaptor can engage the kinase IRAK, that once activated associates with TRAF6 and can induce two signalling pathways JNK or NF-kB (Janeway and Medzhitov, 2002).
Activation of these signalling pathways plays a critical role in the initiation of the adaptive immune responses, as it induces the expression of costimulatory molecules (e.g. CD40, CD80, CD86) on the cell surface, secretion of inflammatory cytokines (e.g. IL-1β, IL6, IL-12, TNF-α) and chemokines receptors (CCR2, CCR5, CCR7) (Akira et al., 2001), that together with MHC molecules is necessary for the activation of naive T cells (Figure 1.3.).

1.3. DCs and T cell priming

T cell activation is a very well regulated process that occurs in secondary lymphoid organs and is crucial in the induction of adaptive immune responses.

T cell priming needs the integration of three fundamental signals derived by DCs:

- **Antigen- loaded MHC complexes**, that activate the T cell receptors (TCR); TCR triggering determines the antigen-specificity of the T cell response;
- **Co-stimulatory molecules**, lead to long-term immunity, as they provide a balance of co-stimulatory and co-inhibitory signals that modulate the T cell response, thus promoting the survival of effector T cells and supporting the differentiation of long-lived memory T cells (Edwards et al., 2002; Greenwald et al., 2005; Watts, 2005). T cell tolerance is mainly a consequence of antigen presentation in the absence of these secondary signals, situation that is dependent on the state of activation of the DCs (Probst et al., 2005);
- **Soluble cytokines**, produced by DCs in different quantity and quality, depending on the conditions of DC priming. The balance of cytokines secreted can induce differential T cell proliferation, survival, differentiation and development of effector functions, i.e. CD8⁺ expanding as cytotoxic T lymphocytes (CTLs) and CD4⁺ differentiating as T helper cells (Th1, Th2, Th17) or as regulatory T cells respectively. The fate of tolerized T cells depends also by the microenvironment; certain forms of inflammation for instance, such as cancer, can change the functional status of DCs from immunogenic to tolerogenic, because of the presence of suppressive factors (Aymeric et al., 2010).

The events that lead to T cell immunity are further influenced by crosstalk with accessory leukocytes, such as natural killer (NK) cells and NKT cells, which interact with DCs and provide additional maturation stimuli that enhance the stimulatory potential of the DCs (Corthay, 2006; Semmling et al., 2010; Turley et al., 2010).
1.3.1. Cytokine secretion by DCs for T cell priming

Cytokine secretion is one of the most important languages of communication between immune cells that determine the type, quality, amplitude, duration and outcome of the immune response. Cytokines also link cells of the immune system to those in surrounding tissues. During development, after injury or in tumour growth, cytokines can pass on destructive or reparative signals to other cells (Salamonsen et al., 2007; Yan and Hansson, 2007).

A fundamental role of DCs is the orchestration of various forms of immunity and tolerance; different maturation stimuli can induce DCs to differentiate and produce specific signals, primarily cytokines, able to induce CD8\(^+\) and CD4\(^+\) T cell proliferation and differentiation towards different functional phenotypes: cytotoxic T lymphocytes (CTLs) or T helper Th1, Th2, Th17 or T regulatory cells respectively.

**CD4\(^+\) T cell priming**

The way in which DCs bias the development of Th cell subsets is related to the specific pattern of stimuli inducing the DC maturation and to the factors produced in the surrounding tissues, which modulate the milieu of cytokines produced by DCs.

- **Th1** cells polarization evolved to enhance clearance of a broad spectrum of intracellular bacteria, viruses and some parasites. Engagement of TLR3, 4, 7 and 9 on DCs by these pathogens results in the production of both types of IFNs and IL-12, which play a crucial role in inducing Th1 cells differentiation and in the cell proliferation and survival of committed Th1 cells. Th1 differentiation is coupled to the sequential action of IFN-\(\gamma\) and IL-12 through a signalling pathway that involves STAT1 and the transcription factor T-bet, which represent the master regulator of Th1 cells (Szabo et al., 2000).

- **Th2** responses are induced by infection with parasitic helminths and various allergens. The key cytokine involved in Th2 differentiation is IL-4 that initiates a signalling pathway via STAT6 and leads to the upregulation of the transcription factor GATA-3. This master regulator of Th2 differentiation induces the transcription of IL4, IL5 and IL13 genes while suppressing the factors critical to Th1 pathway.

- **Th17** cells are a third class of effector CD4\(^+\) T cells that has been implicated in numerous autoimmune and inflammatory conditions and are characterized by production of a distinct profile of effector cytokines, including IL-17 and IL-6. They probably have evolved to enhance the host clearance of a range of pathogens distinct from those targeted by Th1 and Th2. Indeed, IL-17 has been linked to resistance to infection by extracellular bacteria such as *Klebsiella*
pneumoniae as well as by fungi such as Candida albicans. Has been shown that the secretion of proinflammatory cytokines such as TNF and IL-23 strongly bias Th differentiation to Th17 fate. It has also been shown that Th17 differentiation is dependent on the presence of T regulatory cells and requires TGF-β.

- A fourth subset developing from CD4$^+$ T cells is that of regulatory T cells, whose differentiation is induced by cytokines like IL-10 and TGF-β; this subset is characterized by the ability to suppress adaptive T cell functions and prevent the immune response.

**CD8$^+$ T cell priming**

DCs play a crucial role in the generation of CTLs. According to the “licensing” model, CD8$^+$ T cell differentiation towards memory T cells and to effective CTLs needs a previous encounter, by DCs, of antigen-specific CD4$^+$ T cells. IL-4 appears to be essential in this communication (Stager et al., 2003), either in vaccine model or tumour specific response (Schuler et al., 2001), being at the head of a cascade which includes the production of IL-12 in DCs. It has been shown however that DCs activated by TLR3 or TLR9 ligands can trigger the induction of functional, fully differentiated CTLs, without the need of Th cells interactions. This differentiation is triggered by type I IFNs and IL-12 produced by DCs upon TLR-induced maturation.

**1.3.2. T cell priming vs. T cell inhibition: functions of IL-12 and IL-10 on CD8$^+$ T cell priming**

**Interleukine-12 (IL-12)**

As described above, IL-12 is an extremely important cytokine secreted by DCs to induce CD8$^+$ T cell adaptive response to pathogens.

The molecule of IL-12 is a heterodimer formed by a 35-kDa light chain (p35 or IL-12α) and a 40-kDa heavy chain (p40 or IL-12β) (Kobayashi et al., 1989). IL-12 p40 is produced in large excess over the IL-12 in mice, and production of p40 homodimers, not biologically active, has been observed in vivo (Heinzel et al., 1997).

The IL-12 receptor (IL-12R) is composed of two chains (IL-12Rβ1 and IL-12Rβ2) (Presky et al., 1996) that activate (JAK)–STAT (signal transducer and activator of transcription) pathway of signal transduction and STAT4 transcription factor.

IL-12R is expressed mainly by activated T cells and NK cells, but has been shown also on DCs (Grohmann et al., 1998) and B-cell lines (Airoldi et al., 2000).
Activation of Th1 cells through the TCR upregulates the transcription and expression of IL-12R, and this upregulation is enhanced by IL-12 itself, IFN-α, IFN-γ, TNF and co-stimulation through CD28.

The initial production of IL-12 in DCs occurs rapidly, and it has been shown to be independent of IFN-γ and of signals from T cells (Gazzinelli et al., 1994; Scharton-Kersten et al., 1996), even if it is facilitated by stimulation through CD40L (Schulz et al., 2000). Similar to other pro-inflammatory cytokines, the production of IL-12 is regulated strictly by positive and negative regulatory mechanisms. Products from microorganisms — including bacteria, intracellular parasites, fungi, double-stranded RNA, bacterial DNA and CpG-containing oligonucleotides — are strong inducers of IL-12 production by DCs (Ma and Trinchieri, 2001); the relative efficiency of the various inducers depends on the differential expression by DC subsets of the TLRs that these products engage (Jarrossay et al., 2001; Kadowaki et al., 2001).

Various cytokines, such as IFN-γ and IL-4, can increase the ability of cells to produce IL-12 (Hayes et al., 1998). The ability of IFN-γ to enhance production of IL-12 forms a positive-feedback mechanism during inflammatory and Th1 responses. Surprisingly, the Th2 cytokine IL-4 is also potent enhancers of IL-12 production (D’Andrea et al., 1995). Its effect on expression of the gene encoding p40 is bimodal: at early times (<24 hours), it inhibits p40 production, whereas at later times, it strongly enhances it. IL-4 acts by amplifying transcription of the genes encoding both p40 and, in particular, p35, and it augments production of the heterodimer even more efficiently than does IFN-γ (Marshall et al., 1997).

T cells enhance the production of IL-12 not only through their secretion of cytokines, but also through direct cell–cell interactions, best exemplified by the interaction of CD40L on activated T cells with CD40 on DCs in the immunological synapse (Macatonia et al., 1995; Schulz et al., 2000).

IL-10, which is a crucial factor for maintenance of the fine balance between effective resistance against pathogens and detrimental systemic inflammation, is a potent inhibitor of IL-12 production by blocking transcription of both of its encoding genes (Aste-Amezaga et al., 1998). TGF-β is also an inhibitor of IL-12 production that, unlike IL-10, also reduces the stability of IL-12 p40 mRNA (Du and Sriram, 1998).

Although IFN-α and IFN-β have some functions that overlap with those of IL-12 in terms of the induction of IFN-γ production and Th1 responses, and TNF also is strictly interconnected with
IL-12 in regulation of the inflammatory response and IFN-γ production, they suppress IL-12 production (Cousens et al., 1999; Ma and Trinchieri, 2001).

The role of IL-12 in priming T cells during the antigen presentation, is synergistic with the stimulation in the IS via co-stimulatory molecules CD80/CD86 on DC of CD28 on T cell. IL-12 enhances the generation of CD8+ T cells, and it augments the cytotoxic activity of them and of NK cells (Trinchieri, 1998), inducing them to produce several cytokines such as GM-CSF and TNF, apart from IFN-γ, cytotoxic granule-associated molecules, such as perforin and granzymes, and upregulating the expression of adhesion molecules (Chan et al., 1991; Kobayashi et al., 1989; Kubin et al., 1994), resulting in a strong immune response against microbial and tumour antigens.

The importance of IL-12 as an IFN-γ inducer lies not only in its high efficiency at low concentrations, but also in its synergy with many other activating stimuli, especially the cell-cell contact signals exchanged in the immunological synapse (Chan et al., 1992).

**Interleukine-10 (IL-10)**

TLR engagement induces not only pro-inflammatory IL-12 but also anti-inflammatory IL-10 secretion from DCs.

IL-10 modulates expression of cytokines, soluble mediators and cell surface molecules by cells of myeloid origin, with important consequences for their ability to activate and sustain immune and inflammatory responses.

IL-10 plays a key role as feedback regulator in Th2 and Th17 cells, and in regulatory T-cell (Treg) mediated immune suppressive functions (Askenasy et al., 2008; Saraiva and O'Garra, 2010). In addition to IL-10, other molecules known to contribute to immune suppression become active: phosphorylation of STAT3 (Yu et al., 2007), up-regulation of indoleamine- 2,3-dioxygenase (IDO) (Munn et al., 2002). IDO renders activated T-cells susceptible to apoptosis and contributes to Treg activation (Jurgens et al., 2009).

IL-10 have a strong inhibition effect on a many activated macrophage/monocyte functions, including monokine synthesis, NO production, and expression of class II MHC and costimulatory molecules such as CD80/CD86, and these effects lead to the inhibition in cytokine production by both T cells and NK cells.

In the context of APCs, IL-10 potently inhibits production of IL-1, IL-6, IL-10 itself, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF and PAF by activated DCs and macrophages.
The inhibitory effects of IL-10 on IL-1 and TNF production are crucial to its anti-inflammatory activities, because these cytokines often have synergistic activities on inflammatory pathways and processes, and amplify these responses by inducing secondary mediators such as chemokines, prostaglandins, and PAF.

IL-10 inhibits expression of MHC class II antigens, CD54 (ICAM-1), CD80 (B7), and CD86 (B7.2) on monocytes, even following induction of these molecules by IL-4 or IFN, through a posttranscriptional mechanism involving inhibition of transport of mature, peptide loaded MHC class II molecules to the plasma membrane.

Downregulated expression of these molecules significantly affects the T cell–activating capacity of APC. Thus, IL-10 treatment of DC can induce or contribute to a state of anergy in allo-antigen or peptide-antigen activated T cells.

Figure 1.4. Induction of the antimicrobial immune response
1.4. Cytokine release: constitutive and regulated secretion

Even if the knowledge about cytokine actions in the immune system is very well established, just how cytokines are released or secreted by innate immune cells remains a significant “black box” in immunology, and the precise mechanisms of cytokine trafficking and release remain obscure in many cell types.

Our knowledge of cytokine secretion goes in parallel with the field of intracellular trafficking and both have advanced, thanks to new technologies that allow newly-synthesized proteins to be visualized and tracked through cells as fluorescently tagged proteins (e.g. with green fluorescent protein (GFP) and its many derivatives) using imaging in live and fixed cells (Giepmans et al., 2006; Lippincott-Schwartz, 2004). The functional components of the trafficking machinery can now be studied in vitro with RNA interference and expression of mutated proteins, as well as through the use of knockout and transgenic mice.

In classical secretory pathways (the preferential for the cytokine release), newly synthesized cytokines are trafficked to the Golgi complex (GC) and at the trans-Golgi network (TGN), where they can be loaded into vesicles or carriers for immediate release through constitutive secretion. Alternatively, cytokines and other cargo can be loaded into granules, lysosome-related organelles (LROs), or secretory lysosomes and stored until their receptor-mediated release through a regulated secretion (Figure 1.5.).

Like all newly synthesized proteins, most cytokines rely on a very high number of membrane-bound and cytoplasmic cellular proteins, that are called trafficking machinery, which mediate their transport through the cell, allowing their introduction into secretory carriers and the following phases of budding, movement and fusion of the membrane-bound carriers at each transport step (De Matteis and Luini, 2008; Hughson and Reinisch, 2010; Stenmark, 2009).

This complex machinery includes large families of proteins from which individual family members operate in distinct combinations to provide specificity for each transport step. The majority of this machinery resides in the membrane and is assembled at specific sites on organelle or carrier membranes to facilitate the transport of newly synthesized proteins.

Members of the ADP ribosylation factor (ARF)/Sar family of small guanosine triphosphatase (GTPases) and their accessory proteins help to sort and load secretory cargo into coating protein I (COPI) or COPII-coated vesicles for the bi-directional transport between the ER and Golgi (Spang et al., 2010). At the TGN, ARF proteins and adaptor complexes sort some proteins into...
clathrin-coated vesicles for transport to late endosomes, while secretory proteins are commonly loaded into uncoated tubulo-vesicular carriers for constitutive transport to endosomes or directly to the cell surface.

**Figure 1.5. The classical secretory pathway**

Proteins (e.g., cytokines) synthesized in the ER and Golgi complex are transported in membrane-bound vesicles, granules or both, to the cell surface for release. While all cells have constitutive pathways, specialized cell types additionally have regulated (granule-mediated) pathways, and some cells have a variation of this process (piecemeal degranulation) in which membrane-bound vesicles are used to selectively transport cytokines from secretory granules to the cell surface. All of these pathways have multiple transport steps, each requiring sets of trafficking machinery molecules to execute carrier budding, movement and membrane fusion.

Vesicle budding at each step requires membrane curvature for the formation of “buds” or tubules, and fission to release them as carriers. These actions involve series of proteins, lipid kinases, and phosphatases, along with the GTPase dynamin, as well as other fission proteins (De Matteis and Luini, 2008). Both actin- and microtubule-based motors assist in the movement of carriers through the cell (Loubery and Coudrier, 2008; Verhey and Hammond, 2009). The docking and fusion of carriers at target membranes then involve specific combinations of tethering complexes, Rab GTPases (Stenmark, 2009) and SNARE fusion proteins (Jahn and Scheller, 2006).

**Constitutive exocytosis** is the predominant mechanism for cytokine secretion in DCs and macrophages. The original dogma held that constitutively secreted proteins (whose secretion may be induced or not by the activation of a receptor) were trafficked directly from the TGN to the cell surface for release, but after the latest observations obtained thanks even to live cell imaging, it has now been established that many newly synthesized proteins exiting the TGN, prior to being transported to the plasma membrane for release, are delivered to intermediate subcellular compartments such as endosomes (Figure 1.6.). The most well characterized are tubulo-
vesicular “recycling endosomes”, known to be implicated in both endocytic and exocytic pathways in many immune cells (e.g. NK cells, mast cells, macrophages) (Ang et al., 2004; Lock and Stow, 2005).

![Diagram](image)

**Figure 1.6. Sub-cellular trafficking pathways for exocytosis**

Newly synthesized proteins arriving from Golgi apparatus are sorted into TGN towards different destinations. The exit routes from the TGN include those towards the apical plasma membrane, the basolateral plasma membrane, recycling endosomes, early endosomes, late endosomes and specialized compartments such as secretory granules in secretory cells. These are the main destinations, and for each of them more than one type of carrier might be involved. Golgi-resident proteins recycle back to the Golgi stack, as secretion consumes the last trans-Golgi cisternae.

Other two interesting vesicular organelles, which have been associated mostly to the regulated exocytosis of pre-stored granules (whose secretion is induced by the activation of a receptor) but also to the polarized secretion of cytokines (the IL-18 released by iDCs in the IS with NK cells (Semino et al., 2005)) are the late endosomes and the lysosomes. These organelles contain digestive enzymes active at low pH, and are assigned for the degradation of endocytosed molecules and for the secretion of cytotoxic mediators.

Thus, cytokine trafficking is regulated both temporally and spatially to orchestrate immune responses. The mode of release is often optimized to enhance the rapidity or efficiency of cytokine release, to direct cytokines to a target or to maximize their dispersal.
1.5. SNARE proteins: the regulators of vesicular traffic

The fusion between vesicles or the plasma membrane is not a spontaneous event. Since the late 1980s, when Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) were first characterized, rapid progress has identified SNAREs as key elements of membrane fusion, one of the basic reactions of the intracellular trafficking, and now they appear to be implicated in all of the trafficking steps of the secretory pathway (Wilson et al., 1989).

SNAREs are small proteins composed mainly of a C-terminal membrane anchoring domain and an evolutionarily conserved α-helical domain (the SNARE motif) that mediates membrane fusion. Many SNAREs have independently folded domains that are positioned N-terminally to the SNARE motif and that vary between the subgroups of SNAREs. Exceptions to this general definitions are some SNARE subgroups: the evolutionarily younger “brevins”, which lack the N-terminal domain; another subset contains post-translational modifications in the C-terminal domain which mediates the membrane anchorage, and this is the case of the synaptosome-associated proteins (SNAPs), that have two different SNARE motifs joined by a flexible linker that is palmitoylated.

![Figure 1.7. Schematic representation of SNAREs structure and a SNARE complex](image)

*Figure 1.7. Schematic representation of SNAREs structure and a SNARE complex*

*a Domain structures of the SNARE subfamilies. b The four-helix bundle structure is shown as ribbon diagram. c Structure of the central ‘0’ layer.*

Key to understanding the function of SNAREs in membrane fusion was the discovery that different sets of SNAREs that are present in two opposing membranes associate into complexes that are subsequently disassembled by N-ethylmaleimide-sensitive factor (NSF) (Wilson et al., 1989). Complex formation is mediated by the SNARE motifs, and is associated with conformational and free-energy changes. When SNAREs are monomeric, SNARE motifs are unstructured, but when appropriate sets of SNAREs combine each other, the SNARE motifs
spontaneously associate to form helical core complexes of extraordinary stability (Fasshauer, 2003). Core complexes are represented by elongated coiled coils of four intertwined, parallel α-helices, with each helix being provided by a different SNARE motif. The centre of the bundle contains 16 stacked layers of interacting side chains. These layers are largely hydrophobic, except for a central ‘0’ layer that contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue. Accordingly to this contribution to the 0 layer, SNAREs are classified into Qa-, Qb-, Qc- and R-SNAREs (Fasshauer et al., 1998). Functional SNARE complexes that drive membrane fusion are hetero-oligomeric, parallel four-helix bundles, and each bundle is invariant, requiring one of each Qa-, Qb-, Qc- and R-SNAREs (Figure 1.7.).

According to the model in which the assembly of SNAREs would be the driving force behind fusion (Lin and Scheller, 1997), the ‘zippering’ of the SNARE motifs clamps the membranes together and initiates fusion, with SNAREs playing the direct role of fusion catalysts.

It is likely that assembly is an ordered, sequential reaction rather than a random collision of four different SNARE motifs, and this hypothesis takes shape from the observation that SNARE complexes assembly in vitro proceeds through a defined and partially helical Qabc intermediate (Fasshauer and Margittai, 2004; Fiebig et al., 1999), the formation of which is rate limiting. Only when an acceptor scaffold is available in which the N-terminal ends of the SNARE motifs are structured, is the final SNARE able to bind with biologically relevant kinetics and nucleate the zippering reaction.

There are 38 known members of the mammalian SNARE family at present. Functionally, SNAREs are classified as ‘v-SNAREs’ found on the vesicle membrane and ‘t-SNAREs’ found on the target membrane. Vesicular transport is driven by specific interactions between specified v-SNAREs and their cognate t-SNAREs.

SNAREs, particularly vesicle-associated membrane proteins (VAMPs), SNAPs and syntaxins, are specifically localized throughout vesicular compartments, including endoplasmic reticulum (ER), trans-Golgi network (TGN), early endosomes (EE), secretory vesicles, and the plasma membrane (PM) (Lippincott-Schwartz et al., 2000).

Up to now, at least 18 syntaxins and 8 VAMPs have been characterized, which are localized to various subcellular compartments and mediate the docking and fusion of vesicles therein (Jahn and Sudhof, 1999; Mellman and Warren, 2000).
The selective pairing of SNARE proteins directs trafficking and membrane fusion between organelles or with the cell surface.

So, defining the locations and functional partners of individual SNAREs has emerged as a powerful initial approach for mapping intracellular pathways and manipulating both trafficking steps and cellular responses (Huse et al., 2006; Murray et al., 2005b).

1.5.1. SNAREs and cytokine secretion

Each cell type is known to express different combinations of SNARE family members selectively distributed on organelles and membrane domains. Depending on the physiological requirements of each cell type, the repertoire and function of its SNARE proteins can be as diverse as the transport of receptors to and from the cell surface, constitutive- and induced-secretion of immune and inflammatory mediators and the release of neurotransmitters (Logan et al., 2003; Wang and Tang, 2006).

In the last decade, the involvement of SNARE molecules in cytokine release of immune cells has been focus of investigations (Moqbel and Coughlin, 2006; Stow et al., 2006).

Figure 1.8. Schematic representation of SNAREs implicated in the secretory pathways in immune cells

The R-SNAREs involved in regulated and constitutive exocytosis are distinct, with VAMP-2, -7, and -8 implicated in regulated secretion, whereas Vti1b and VAMP-3 are essential for constitutive release of recycling endosomes. A number of GTPases are also associated with cytokine release.

Various SNARE complexes have been identified which have helped to identify the intracellular pathways leading to the secretion of cytokines in different cell types.
<table>
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<th>Cell type</th>
<th>SNARE partners</th>
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<td>Plasmas</td>
<td>Cells</td>
<td>ND</td>
<td>Antibody secretion</td>
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Table 1.1. SNARE proteins and complexes known to mediate immune functions

*One or more of the partner SNAREs is unidentified for this SNARE. †Whether STX6 is present in neutrophils is unclear. ND, not determined; SNAP, soluble-N-ethylmaleimide-sensitive-factor accessory protein; SNARE, SNAP receptor; STX, syntaxin; TNF, tumour-necrosis factor; VAMP, vesicle-associated membrane protein; Vti1b, vesicle transport through interaction with t-SNAREs homologue 1b.

The Q-SNARE proteins syntaxin 6 (Stx6), syntaxin 7 (Stx7) and vesicle transport through interaction with t-SNAREs 1B (Vti1b) for instance, have been found colocalized in the Golgi complex of macrophages with newly synthesized TNF-α precursors (Murray et al., 2005b). Upon LPS or IFN-γ stimuli, TNF-α is translocated to plasma membrane where it co-localizes with syntaxin 4 and SNAP-23, that mediate its release through the phagocytic cup (Pagan et al., 2003). This translocation has an intermediate fusion step with the recycling endosomes, and the
R-SNARE which modulates the two fusion steps, by forming SNARE complexes with the Golgi complex- and the plasma membrane- Q-SNAREs is VAMP3.

VAMP3 has also been identified as mediator of the IL-6 transport in macrophages, which, for many aspects, is superimposable to that of TNF-α, with the exception consisting in a more generalized release through the plasma membrane (Manderson et al., 2007). In other hematopoietic cells such as granulocytes (neutrophils, eosinophils and mast cells) and platelets, a wide range of factors and immune mediators are stored in pre-formed granules; SNARE complexes on these secretory granules mediate their regulated secretion (Table 1.1.).

Few examples are the Stx2-SNAP-23 complex on platelet granules, interacting either with VAMP3 or VAMP8 to selectively modulate the fusion of different subgroups of granules (Chen et al., 2000; Feng et al., 2002), and the Stx4-SNAP-23 complex for the different subgroups of eosinophilic granules, binding differentially and selectively the R-SNAREs VAMP2, VAMP7 and VAMP8 (Logan et al., 2002; Logan et al., 2006).

The complex Stx4-SNAP-23 is found implicated also in the release of azurophilic granules to the phagocytic cup in activated neutrophils. In this case the R-SNARE modulating the fusion to the plasma membrane is VAMP7 (Logan et al., 2006).

Marcet-Palacios and colleagues showed that VAMP7 is also a crucial component of granzyme B release and targets cell killing in NK cells (Marcet-Palacios et al., 2008).

A separate chapter will be dedicated to the current knowledge about targeted secretion in DCs and T cells and relative SNAREs implicated in delivery of different cytokines and mediators.

1.5.2. Rab GTPases: the engines of the traffic

Intracellular trafficking requires tight coordination between the formation of the transport carriers from a donor membrane, their transport along cytoskeletal tracks, and their anchoring and fusion to the correct acceptor membrane.

Particularly important for the specification of intracellular traffic is the Rab family of small GTPases. Rab proteins function by recruiting various effector molecules and tethering complexes that specify the identity and the trafficking activity of the different vesicles (Grosshans et al., 2006; Zerial and McBride, 2001) (Figure 1.9.).
Over 60 Rab proteins have been identified in mammals, and each defines a distinct pool of vesicles. Like other regulatory GTPases, the Rab proteins switch between two different conformations, one GTP-bound regarded as active, and the other GDP-bound, the inactive form. The GTP-bound form is the one that interacts with downstream effector proteins (Zerial and McBride, 2001).

Directionality and efficacy of vesicle delivery are in part mediated by actin filaments and microtubules, which facilitate local and long-range vesicle transport, respectively. Higher eukaryotes contain various motor proteins that are capable of powering directional vesicle transport along such molecular cables. This necessitates high specificity in the attachment of motors to vesicles, and there are several examples in which Rab GTPases and their effectors proofread these types of interactions.
Rab GTPases also work with SNAREs to align docking and fusion of vesicles at the target membranes; few examples are IFNγ and TNF-vesicular trafficking in macrophages (Wickner, 2010).

All Rabs so far characterized are associated with specific organelles, where they act as targeting determinants for a wide variety of protein, including molecular motors and tethering factors (Pfeffer, 2001).

For example, the ubiquitous Rab6 localizes to the trans-Golgi where it recruits Bicaudal D, an accessory protein for the microtubule motor dynein (Matanis et al., 2002; Short et al., 2002), and TMF1, a coiled-coil protein involved in membrane traffic (Fridmann-Sirkis et al., 2004).

Rab5 recruits many proteins to early endosomes including EEA1, a tethering factor for endosome fusion (Simonsen et al., 1998); Rab 7 and Rab11 associate to late and recycling endosomes respectively.

By contrast, Rab27a has been found implicated in lysosomal secretion in CTLs and melanocytes, where it mediates the release of lytic granules and melanosomes respectively, mediating anchoring to the actin cytoskeleton (Seabra and Coudrier, 2004). Functional loss of this protein indeed is cause of the development of the Griscelli syndrome, characterized by albinism and defects in the cytotoxicity of CTLs and NK cells (Menasche et al., 2000).

Other diseases have been associated to loss of Rabs and their regulators or effectors, thus reflecting the physiological importance of Rab GTPases. In particular, infectious, neurological and endocrinological diseases can result from pathogen-induced or inherited dysfunctions of Rab pathways, consistent with the crucial role for membrane trafficking in immunity and regulated exocytosis.

The involvement of Rab GTPases in cell signalling is reflected also by the association of Rab dysfunctions with cancers. Dysregulated receptor signalling and trafficking is associated with cancer development (Bache et al., 2004; Polo et al., 2004), and therefore the aberrant expression of Rab GTPases might be predicted to cause tumorigenesis.

Indeed, Rab25 is frequently overexpressed in breast and ovarian cancers and is strongly associated with decreased survival (Cheng et al., 2004).

Other Rab GTPases that have been implicated in carcinogenesis include Rab8 and Rab23 (Eggenschwiler et al., 2001; Hou et al., 2008).
Because of the recent opportunities provided by microarray analyses and high-throughput sequencing of tumours, additional Rab GTPases involved in cancer, as well as their regulators and effectors, are likely to be identified in the near future.

1.6. DC-T cell contacts: the Immunological Synapse

As described above, DCs can induce T cell priming through three distinct signals: antigen loaded on MHC complexes and presented at the plasma membrane, co-stimulatory molecules on cell surface, and release of soluble mediators such as cytokines and chemokines.

In order to present antigens and co-stimulatory molecules for the activation of T cells, DCs must create a physical connection with T lymphocytes, by forming specialized signalling areas constituted by cell–cell adhesive junctions. These areas are called immunological synapses (IS) (Dustin and Colman, 2002; Valitutti, 2008).

T cell – IS

IS was first characterized in 1998 by Kupfer and colleagues, observing the molecular pattern expression by the point of view of the T cells. They defined micrometer-scale molecular structures that were called supramolecular activation clusters (SMACs) (Monks et al., 1998), formed very early after T cell-APC (they used B cells) conjugation and composed of two concentric regions: the central SMAC (cSMAC) enriched in T cell receptors (TCRs) and the peripheral SMAC (pSMAC), a ring with high concentration of integrins LFA-1 and their ligands ICAM-1 (Figure 1.10.).

However, data collected from experiments performed in T cells and DCs suggest a more complex organization of T cells surface proteins at the IS (Dustin, 2009). It is believed that these protein patterns are stabilized on the membrane by the insertion of these surface protein components in rafts and/or tetraspanins domains and/or through interaction of surface proteins with the cytoskeleton (Barreiro et al., 2007; Miceli et al., 2001).

With regard to the cytoskeletal proteins located in the cytoplasmic regions of the T cell at the IS, we find actin and tubulin, and cytoskeletal molecules associated to both. These proteins are important to preserve the structural integrity of IS and to provide scaffoldings for the signalling complexes assembling in these regions (Dustin, 2009; Huppa and Davis, 2003).

Besides the cytoskeletal molecules, at the cytoplasmic side of the IS in T cells there are arrays of signalling molecules that facilitate the spreading of the signals from the surface receptors, e.g.
different types of kinases (e.g. Akt, PKC). Other proteins also contribute to the organization and to the transmission of intracellular signals in these areas, such as adaptors (e.g. Dlg1), small GTPases (e.g. Rac1, Rho) and guanine nucleotide exchange factors (GEFs) - which together control the architecture of these regions- and some different deacetylases which regulate the tubulin cytoskeleton.

It has also been proposed that the IS may control the endocytosis of the T cell receptor (TCR) for the termination of its signalling. Another hypothesis is that the IS formation may modulate the activity of the TCR by boosting low and lowering high-intensity signals arriving to it.

In summary, the molecular characteristics of the immunological synapse in T cells show that this is an active signalling region that controls important immune functions related to the adaptive immune response.

As already said, the IS is a zone with very high exchange of signals between the two cells that are in contact, and there are many molecules that recognize each other and bind together to get the signalling started. TCRs for instance recognize MHC complexes loaded with antigens and present on the cell surface of the DC. The different affinity to binding MHC class I or class II defines respectively CD8 and CD4 T cells. Ligand recognition by TCR causes the T cell to stop migrating and to form an increasingly stable cell contact with the corresponding dendritic cell, also through the integrin-ligand interactions (ICAM-1), which allow tight contacts between T cells and DCs, necessary to stabilize the IS (Davis, 2009). This mechanism involves the
reorientation of the MTOC (as well as its associated molecules), and the recruitment of receptors and signalling molecules to the forming immunological synapse (Huppa and Davis, 2003).

**DC – IS**

The knowledge on the structure and functions of the IS in DCs is still sparse, if compared to the large amount of informations available on the IS by the T cell point of view (Rodriguez-Fernandez and Corbi, 2005).

The structure of the T cell–DC IS has been a matter of debate, and evidence is emerging that it diverges from the “bull’s eye pattern” established for T cell–B cell interactions. It has been proposed indeed that the DC cytoskeleton plays a peculiar active role in defining the T cell–DC immunological synapse; DC maturation induce a huge rearrangement of the cytoskeleton, thus allowing the formation of more stable contacts with naïve T cells and mature IS (Benvenuti et al., 2004a) and it has been shown that the DC cytoskeleton can modify the location of key molecules (Dustin et al., 2006).

Evidence show that proteins involved in the IS area in DCs may have a similar pattern with respect to T cells in the same region. In this regard, the IS of DCs, also includes surface proteins that are associated to cytoplasmic structural and signalling molecules, and many of those reflect oppose receptors on the T cell surface in IS (Barreiro et al., 2007).

Regarding the composition of cytoplasmic regions of the IS area in DCs, the observation of *F-actin clusters* suggests that cytoskeletal changes are necessary to maintain the structure and probably the signalling from this region (Al-Alwan et al., 2001; Riol-Blanco et al., 2009), and this hypothesis has been reinforced by the observation that disruption of the F-actin in DCs by using cytochalasin D blocks IS formation (Al-Alwan et al., 2001), and also that clusters of *phosphatidylinositol 4,5-bisphosphate* (PI(4,5)P2), a lipid kinase product that modulates the actin cytoskeleton, are found at the IS (Fooksman et al., 2009).

IS is also an important region of active signalling, and this is proven by the presence of tyrosine phosphorylated proteins accumulating in this area. In this regard, upon IS formation, kinases like *Akt* are mobilized to this zone (Riol-Blanco et al., 2009). The identification of *spinophilin*, a PDZ containing adaptor previously described at the neural synapse, indicates that, similarly to the T cell pattern, adaptors may be key signalling component in DCs at the immunological synapse.

Finally, the fact that disruption of the IS formation blocked T cell activation further emphasizes the importance of the DC molecular pattern in this zone (Al-Alwan et al., 2001).
IS formation in vivo

Despite the characterization of the mechanisms leading to T cell activation, the way individual T cells experience their encounters with DCs in vivo in secondary lymphoid organs has remained largely unknown until the introduction, in 2002, of two photon microscopy technology, which have allowed the visualization of the dynamics of T cell activation in a native environment at single-cell resolution (Miller et al., 2002). Time-lapse videos have been produced by visualizing fluorescently labelled T cell and DCs interacting in explanted lymphnodes or also in intravital preparations from anesthetized mice, revealing the diversity of Tcell-DC contact dynamics during antigen recognition.

This diversity consists in the duration, stability and number of cell-cell contacts that can be formed between APC and T cells, and it regulate the efficiency of T cell priming (Bousso and Robey, 2003; Hugues et al., 2004; Shakhar et al., 2005); these parameters are dependent on the state of maturation of DCs (Benvenuti et al., 2004b), on pMHC concentration on DC cell surface (Bousso and Robey, 2003), on the presence in the environment of different milieu of cytokine and chemokines, or of different subsets of T lymphocytes. T reg for instance inhibit the formation of long-lived contacts, whereas presence of cognate CD4⁺ helper T cells favours the formation of contacts between DCs and CD8⁺ T cells (Beuneu et al., 2006).

From in vivo studies, it has been described that in CD4⁺ T cell priming, the minimum interaction time to induce T cell proliferation is 6 hrs, and longer stimulations lead to more robust clonal expansion. The production of IFN-γ in T cells need instead an interaction of several days (Celli et al., 2007).

Mempel and colleagues, utilizing two-photon microscopy on popliteal LNs of anaesthetized mice, have shown a three phase model of CD8⁺ T cell priming in vivo on synchronized resident T lymphocytes. First, DCs in lymphnodes form multiple short lasting contacts with high motile T cells for the first 8 hrs, during which T cell start to decrease their motility and upregulating activation markers. Second, in the next 12 hrs they engage in long lasting contacts and start secreting IL-2 and IFN-γ. Third, the second day T cells start to proliferate and resume their high motility and short lasting DC contacts. The duration of contacts is dependent on the presence of antigen-bearing DCs (Mempel et al., 2004).

Thus, efficiency of priming is very well correlated with the capacity of DCs to form stable contacts with T cells. These contacts produce the conformational rearrangement of both DC and T cells, that are identified as immunological synapse.
All these observations lead to the conclusion that IS is a specific, very well regulated and fundamental structure for bidirectional signalling between DCs and T cells.

1.7. Polarity and cytokine secretion in the IS

During synapse formation, cells involved in the contact undergo a deep process of reorganization, which is functional to the proper communication through the exchange of physical and soluble signals.

It was observed in a number of systems that antigen receptor engagement reverses T-cell polarity and increases T cell adhesiveness. This was first observed in terms of movement of the microtubule-organizing centre (MTOC), which was used as a marker for the secretory apparatus, as is also often positioned at the focus of retrograde actin flow near the uropod in a migrating cell (Dustin et al., 1997). The MTOC was observed to translocate to the centre of the interface between cytotoxic T cells and target cells, and this behaviour was later observed also in helper T cells (Geiger et al., 1982; Poenie et al., 2004).

In 2001 Stinchcombe and colleagues showed that during the encounter with target cells, the centrosome of CTLs polarizes in an actin-dependent manner towards the IS, positioning near the cSMAC region reach of TCRs. This reorientation brings in proximity to the plasma membrane of the IS lysosomal organelles for the rapid release of the lytic granules, and the Golgi complex, thus transporting newly synthesized molecules; this process allows a very well precise and regulated secretion of cytotoxic mediators (Stinchcombe et al., 2001).

In 2006 Huse et al, showed that in T helper cells distinct pathways for polarized release of different cytokines are present that can be secreted either at the immunological synapse or elsewhere on the surface. Cytokines such as IL-2, IFN-γ and IL-10 are secreted into the synapse to communicate with APC in an antigen-specific way that is dependent on microtubule reorganization, whereas factors like TNF, IL-4 and CCL3 are secreted from other points on the cell surface and in a multi-directional way to promote an inflammatory response by surrounding cells, thus indicating the presence of a double way of release in T cell. Furthermore in this study they performed some co-localization studies with trafficking proteins, discovering a differential pattern of SNAREs and Rab proteins interacting preferentially with cytokines released in the synapse or multidirectionally (Huse et al., 2006).
In a study about TCR accumulation at the IS in T cells, IS has been recognized as an active fusion site also for vesicles coming from recycling endosomes (REs). In this study REs polarize towards the contact area between T cell and APCs in an antigen-dependent manner, through the docking between the plasma membrane t-SNAREs syntaxin4 and SNAP23, and the recycling endosomal v-SNARE VAMP3 (Das et al., 2004).

Another function described in T helper cells is the activation of DCs for the IL-12 secretion and the subsequent priming of CD8$^+$ T cells. Bertrand and colleagues showed indeed that early upon TCR engagement with antigen-bearing DCs, Tt1 cell-secretory machinery is polarized towards the IS in a PKCζ-dependent manner, and this polarization triggers the synaptic delivery of IFN-γ and CD40L towards the DC, thus inducing the synthesis and secretion of IL-12 (Bertrand et al., 2010).

Compared to the knowledge present about polarized secretion in T cells, very little is known about the cytokine secretion pathways in DCs during IS formation. At molecular level, the actin cytoskeleton remodelling was identified as fundamental for a proper synapse formation and subsequent efficient T cell priming. Rho GTPases have shown particular importance in these processes: Rac1 and Rac2 have been implicated in the IS formation between mature DCs and CD4$^+$ T cells, through initiation and consolidation of cell contact (Benvenuti et al., 2004a); in other studies the Wiskott-Aldrich syndrome protein (WASp) was identified fundamental for the synapse formation and CD4$^+$ and CD8$^+$ T-cell activation by DCs (Bouma et al., 2007; Pulecio et al., 2008).

More recent evidences showed that in DCs the IS formation triggers a polarization of the MTOC in the direction of the T cell contact site. This polarization is dependent on antigen-specificity of the synapses and on the maturation state of the DCs, and is important for the optimal transmission of soluble cytokines necessary for an efficient CD8$^+$ T cell priming. In this regard, antigen-specific MTOC polarization was shown to redistribute the secretory apparatus towards the DC-T cell contact region. According to this new observations, newly synthesized IL-12 is reoriented towards the T cell very early upon IS formation, in a micro tubule-dependent manner, and the release of the cytokine at the IS results in a more effective T cell priming of CD8$^+$ T cells, detected as increased IFN-γ secretion in antigen-specific synapses and in an upregulation of the activation-marker pSTAT4 already after 30 minutes of DC-T cell interaction (Pulecio et al., 2010).
This results on the communication DC-T define the IS as a centre of bi-directional signalling, in which exchange not only of cell-surface signals, but also of soluble mediators occurs.
1.8. Immune response modulation during cancer development

The development of cancer is the result of a well orchestrated self defence carried out by tumour cells. This defence consists in the escape from the attack of immune cells, thus, disrupting the host immune system, which is progressively suppressed as a result of tumour progression and metastasis (Zou, 2005).

Several important mechanisms used by cancer cells to evade the host immune system have been documented. At present, the most relevant strategies identified involve loss of tumour antigen, alteration of HLA class I antigen (Giorda et al., 2003), lack of costimulation (Byrne and Halliday, 2003), immunosuppressive cytokines (Toutirais et al., 2003), immunosuppressive T cells (Somasundaram et al., 2002) and accumulation of myeloid-derived suppressor cells (Figure 1.11.).

During tumour growth, a large number of different immune cells are recruited and infiltrate the tumour mass or stroma. Due to a redundancy of cell-surface markers between many of the myeloid derived cell subpopulations present in this area, is still not easy to characterize the exact composition of this immune cells.

To summarize the recent results obtained in the field, in the primary tumour site the majority of immune cells are of myeloid origin and express the marker CD11b. The CD11b− fraction is composed essentially of lymphocytes and NK cells, whereas the positive fraction can be divided basically into 3 subtypes (Kerkar et al., 2011):

- CD11c−I-Ab+ DCs,
- F4/80+ macrophages (usually CD11clow, but they can express high levels of CD11c in some exceptions as in the case of the lungs),
- CD11c low F4/80low myeloid cells

**Myeloid-derived suppressor cells** (MDSCs), have been investigated in recent years as a potential link between inflammation and tumour progression, as their activity is characterized by immunosuppression and tolerance, induction of mutations in the tumour microenvironment, promotion of angiogenesis and metastasis, direct support of both neoplastic growth and inflammatory reaction.

In healthy individuals, **immature myeloid cells** (IMCs) generated in bone marrow quickly differentiate into mature granulocytes, macrophages or dendritic cells (DCs).
On the other hand in pathological conditions such as cancer, various infectious diseases, sepsis, trauma, bone marrow transplantation or some autoimmune disorders, a partial block in the differentiation of iMCs into mature myeloid cells results in an expansion of this population. Importantly, the activation of these cells in a pathological context results in the upregulated expression of immune suppressive factors such as arginase (encoded by \textit{ARG1}) and inducible nitric oxide synthase (iNOS; also known as NOS2) and an increase in the production of NO (nitric oxide) and reactive oxygen species (ROS). Together, this results in the expansion of an iMC population that has immune suppressive activity.

This heterogeneous and still not well defined cell population is discriminated by a complex expression pattern of surface markers. The general phenotype of murine MDSCs is defined by the following markers: CD11b$^+$, Gr-1$^-$, F4/80$^{\text{int}}$, CD11c$^{\text{low}}$, MHCII$^{-/\text{low}}$ and CD115$^+$ (Bronte et al., 1998), but new markers and subpopulations keep on emerging.

At present MDSC have been subdivided into two different groups, on the base of their phenotype and differentiation potential:

- **M-MDSCs**, cells with morphology and phenotype ($CD11b^+\text{Ly6C}^{\text{high}}\text{Ly6G}^{\text{low}}\text{SSC}^{\text{low}}$) typical for monocytes, consist of myeloid cells with the ability to differentiate to macrophages (TAM) and DCs with an immature phenotype (iDCs);

- **G-MDSCs**, cells with morphology and phenotype ($CD11b^+\text{Ly6C}^{\text{low}}\text{Ly6G}^{+}\text{SSC}^{\text{high}}$) typical for granulocytes (Youn et al., 2008). These cells are the largest population of MDSCs in tumor-bearing mice, representing $>75\%$ of all MDSCs. They suppress antigen-specific T cell responses, primarily via release of ROS (whereas M-MDSCs produce prevalently NO).

### 1.8.1. Tumour microenvironment affects DC functions

Tumour stroma contains multiple cell types, including endothelial cells, fibroblasts, adipocytes, lymphocytes, macrophages, MDSCs and tumour-infiltrating dendritic cells (TIDC).

While TIDC are present in virtually all human cancers and experimental tumour models, the tumour microenvironment compromises their differentiation, maturation and survival (Zou, 2005). Tumours release a large number of soluble mediators in the tumour microenvironment such as \textit{M-CSF/IL-6, VEGF, TGF-\beta}, that contribute to impairing DC functions, mainly through the activation of signal transduction and activator of transcription \textit{STAT3} (Kortylewski et al., 2005; Pardoll and Allison, 2004) (*Figure 1.11*). In addition, the release of \textit{prostaglandin E2} (PGE2) from tumour cells abrogates DC-mediated T cell priming through an \textit{IL-10}-dependent reduction of TIDC and DC maturation as well as a \textit{CCR7}-dependent DC migration in lymph nodes that induces abortive CD8$^+$ T cell responses (Ahmadi et al., 2008).
Even if it is established that the defects in DC function in cancer are mediated by various tumour-derived factors, the mechanism by which these factors affect DCs remains unclear.

It has been shown that in patients with head and neck squamous cell carcinoma (HNSCC), pDC have a diminished ability to produce IFN-α in response to CpG motif containing oligonucleotide (Hartmann et al., 2003). The tumour-induced down-regulation of TLR9 was proposed as one mechanism contributing to impaired pDC function within the tumour environment.

In another study, a fraction of DCs from tumour-bearing mice and cancer patients were shown to accumulate triglycerides as compared with DCs from tumour-free mice and healthy individuals. DCs with high lipid content were not able to effectively stimulate allogeneic T cells or present tumour-associated antigens, and the administration of an inhibitor of acetyl-CoA carboxylase to tumour-bearing mice in order to normalize lipid content in DC restored their functional activity and substantially enhanced the effects of cancer vaccines (Herber et al., 2010).

In 2005 Ghiringhelli and colleagues showed that in mice and rats bearing B16 melanoma, tumour progression induces an accumulation of CD11c+/CD11b+ DCs with an immature phenotype MHC class II<sup>low</sup>/CD80<sup>low</sup>/CD86<sup>low</sup> (IMDC) in draining lymphnodes and spleen, and
that these cells stimulate the proliferation of regulatory T cells in an MHC II-restricted and TGF-β-dependent fashion (Ghiringhelli et al., 2005).

1.8.2. DCs and immunotherapy

The immune system has the potential to eliminate tumour cells; however this potential is impaired and abrogated by many mechanisms taking place during the establishment of the tumour, and that depend on a milieu of inhibitory molecules secreted by the tumour itself, on the low immunogenicity of neoplastic cells and on the tumour stroma, that develops as a tolerogenic environment which advantages the restiveness and the anergy of immune cells.

Studies on mouse models have shown that the generation of protective anti-tumour immunity is highly dependent on the presentation of tumour antigens by DCs (Diamond et al., 2011; Fuertes et al., 2011). Thus, vaccination strategies involving functional DCs have been developed owing to the special properties of these cells in coordinating innate and adaptive immune responses.

DC vaccination aims at inducing tumour-specific effector T cells able to fight tumour cells. In this process, the first step is to provide DCs with tumour-specific antigens. This can be achieved either by culturing ex vivo DCs that have been derived from patients with an adjuvant (that induces DC maturation) and the tumour-specific antigen, and then injecting these cells back into the patient, or by inducing DCs to take up the tumour-specific antigen in vivo. The adoptive transfer of tumour peptide-pulsed DCs has been found to mediate CD8⁺ cytotoxic T cell responses, thus leading to the regression of established mouse carcinoma (Mayordomo et al., 1995; Zitvogel et al., 1996). Moreover, DCs adoptively transferred into mice bearing MHC class I-negative tumours, promoted NK cell-dependent anti-tumour effects (Fernandez et al., 1999).

However these immunotherapeutic strategies are not so encouraging, due to the inability of in vivo transferred DC to overcome tumour-induced immunosuppression and/or to actually reach the draining lymph node to trigger a long-lasting adaptive immune response (de Vries et al., 2005; Morse et al., 1999).

Dendritic cell exosomes expressing functional MHC molecules and co-stimulatory effectors were shown to prevent tumour outgrowth in a T cell-dependent manner (Zitvogel et al., 1998). Another approach utilized to harness DCs against tumours in vivo relies on NKT cells that recognize glycolipids. Activated NKT cells engage CD1d on DCs and thus mimic the effects of TLR triggering or CD40 stimulation and elicit protective CD4⁺ and CD8⁺ T cell resistance to tumours (Fujii et al., 2004).
Another useful approach is mobilization of different DC subsets; the coordinated regulation of a pDC and CD8α⁺ DC network could dramatically enhance host immunity in mice; functionalized biomaterials incorporating GM-CSF, CpG-ODN and tumour lysates were implanted into tumour-bearing mice of, thus inducing activation of host DCs at the vaccine site and triggering the generation of potent CTLs, resulting in protective anti-tumour immunity. This vaccination strategy not only maintains local and systemic CTL responses for extended periods, but also inhibits FoxP3 Treg activity in vivo, culminating in complete regression of established melanoma tumours. Interestingly, this orchestration of DC network has been shown to occur concomitantly to DC type-I IFN and IL-12 secretion (Ali et al., 2009).

The potent efficiency of the interplay between pro-inflammatory cytokines and DCs have been shown also in other protocols; GM-CSF efficiently induced dendritic cells activation in vivo, thus promoting long lasting Th1 and CD8+ T cell responses (Dranoff et al., 1993). Besides, IL-12 used as an adjuvant to immunisation with a mutant peptide of p53 is a potent activator of Th1 and CD8+ T cells, mediating complete tumour regression in an animal model (Noguchi et al., 1995).

Furthermore, a recent study addressed the function of DCs in an inducible model of ovarian cancer, showing that DCs have a strong and divergent impact depending on tumour progression, being protective at early stages promoting tumour growth at later stages. This further highlights the need of identifying key steps that induce conversion of “good” DCs into “bad” DCs (Scarlett et al., 2012).

In a recent work on human patients, tumour-specific CD8⁺T cells engineered to secrete the inflammatory cytokine IL-12 were shown to trigger a programmatic change in MDSCs, macrophages, and dendritic cells within established B16 melanoma, and this triggering enables the licensed recognition of cross-presented antigen. In addition, the cross-talk between T cells and professional APCs in the presence of an acute inflammatory environment triggered by IL-12 within the tumour microenvironment is critical in causing the regression of large established tumours (Kerkar et al., 2011).

All these approaches underline the importance of restore DCs functionality in terms of antigen presentation and secretion of the potent immunostimulatory cytokine IL-12 for an efficient anti-tumour response.

Therefore, a wider understanding of proteins regulating DC phenotype and cytokine secretion may lead to new approaches for restore immunity in cancer environment. A lot of road still remains to be covered.
AIMS

DCs play a crucial role in the induction of the adaptive immunity and in the maintenance of the peripheral tolerance to self and non-pathogenic environmental antigens. DCs induce T cell responses through signals present on their cell surface, represented by antigen-bound MHC complexes and co-stimulatory molecules, which interact specifically with the naive T cell in the geographically delimited space of the IS. In addition to these two signals, DCs can modulate the T cell response by secreting large amounts of soluble cytokines in response to the activation of TLRs.

Previous studies in my lab showed that DCs maturation by TLR agonist induces the formation of stable DC-T conjugates in which DCs polarize the microtubules cytoskeleton and intracellular secretory machinery towards the IS (Benvenuti et al., 2004b; Pulecio et al., 2010). Furthermore, it has been shown that vesicles carrying the inflammatory cytokine IL-12, a key T cell priming mediator, are found recruited at the IS very early after the encounter with the T cell in antigen-specific synapses, and that this polarization improves the T cell priming efficiency by increasing T cell proliferation and production of IFN-γ.

These observations raised the question of the identity of the vesicles that carry IL-12 at the IS and of the pathways of IL-12 secretion in general. Indeed, despite the biological relevance of IL-12 in shaping the immune response, little is known on the intracellular trafficking routes that control its secretion.

SNAREs are an important family of proteins identified as key elements of all the membrane fusion processes. More specifically, they are implied in all the trafficking steps of the secretory pathways. They have been investigated in many different cell types in order to characterize the intracellular pathways involved in the vesicle-mediated exocytosis of soluble mediators.

- The first aim of my thesis was to investigate the intracellular pathway followed by IL-12 during its secretion. For this purpose I focused on the identification of the SNARE proteins involved in its trafficking to the IS, in order to characterize the vesicular compartments implicated in its targeted release.

- The second goal of my work was to study DCs in a pathological condition: the tumour-induced immunosuppression. In this context I investigated SNARE expression in regulatory DCs, characterized by a strong reduction in the IL-12 secretion capacity.
2. Materials and Methods
**Materials and Methods**

**Mice**

Six to eight week old C57BL/6 mice were purchased from Harlan. OVA-specific, TCR transgenic OT-I mice were purchased from the Jackson Immuno Research Laboratories.

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 mice using culture medium IMDM (Gibco BRL) containing 10% FBS, 50uM β-mercapto ethanol and Primocin, supplemented with Fms-like tyrosine kinase 3 ligand (Flt3L) or with granulocyte-macrophage colony-stimulating factor (GM-CSF).

BM-DCs from previously described VAMP7 KO (Danglot et al., 2012) mice were provided by C. Hivroz, Institute Curie, Paris, FR and grown as above.

Dendritic cells were used for experiments between day 7 and 8, when expression of CD11c was higher than 80%. BM-conventional DC were obtained by negative selection using B220+ microbeads (Miltenyi Biotec).

For isolation of DCs from organs, cell suspension were obtained by digestion with Collagenase D (1.6 mg/ml; Roche) and DNase I (0.1 mg/ml; Roche) for 30 min at 37°C.

Splenic DCs were enriched from total spleen cells by positive selection using CD11c microbeads (Miltenyi Biotec). Lung CD11c+/MHCIIhi DCs were isolated from CD11c+ pre-enriched lung cell suspensions using a FACSaria III cell sorter (BD Biosciences). Purity was higher that 90%. OT-I cells were isolated from total lymph nodes suspension by negative selection using CD8 purification kit (Miltenyi Biotec).

The metastatic 3LL-D122 cell line was cultured in RPMI medium (Gibco BRL) supplemented with 10% fetal bovine serum and 100U/ml gentamycin. Cells were grown onto 75 mm² cell culture-treated flasks and grown for 2 days before collection for in vitro and in vivo experiments.
**Materials and Methods**

**TLR agonists**

The CpG-B (1826) oligonucleotide was from InVivoGen, San Diego, CA. The lipopolysaccharide (*Escherichia coli* O55:B5) was from Enzo Life Sciences, Inc.

**Molecular cloning**

SNAREs sequences cloned into an EGFP plasmid (Syntaxin6 and VAMP3 in -C2 vector with the GFP at the N-terminal, VAMP7 in -N1 vector with the GFP at the C-terminal) were a kind gift of J. Stow (University of Maryland, Brisbane).

For the in vitro transcription compatibility, constructs were further subcloned into pcDNA3.1+ vectors bringing a T7 promoter at N-terminus of the proteins sequences. Then plasmids were linearized cutting a site at the C-terminus in the non-coding region.

P35-GFP was obtained by subcloning a recombinant p35 murine sequence (pORF5-mp35 purchased from Invivogen) in a pcDNA3.1+ together with a GFP sequence at the C-terminal. A nucleotidic linker was kept between p35 and GFP sequences.

**Antibodies and FACS reagents**

The following antibodies for FACS analysis were purchased from BioLegend: FITC-, PE- and PE-Cy7-conjugated anti-CD11c, FITC- and PE-Cy5-conjugated anti I-A^b^, PE- and PE-Cy5-conjugated anti-CD86, PE-Cy5-conjugated anti-CD40, PE- and FITC-conjugated anti B220, PE- and APC-conjugated anti CD11b, PE-Cy5-conjugated anti CD8, FITC- and PE-conjugated anti CD45, FITC-conjugated anti Gr-1. Dead markers PE-Cy5-7AAD and FITC-Annexin V were purchased from eBioscience and Roche respectively.

Stained cells were acquired with a FACSaria III flow cytometer and analyzed with FLOWJO software (version 4.5.4; Tree Star Inc.).
**Materials and Methods**

**RNA isolation, RT-PCR and qRT-PCR**

Total RNA from isolated endogenous DCs of pooled mice, BM-cDCs or silenced-BM-cDCs was isolated by TRI Reagent Isolation Kit (Sigma-Aldrich), following manufacturer’s instructions. DNA was removed from isolated RNA fraction by a treatment with RNAse-free DNase I (Fermentas Inc, Massachusetts, USA). Total RNA was retro-transcribed to cDNA by Moloney murine leukaemia RT (M-MLV-RT) in the presence of random hexamers (IDT). qRT-PCR was based on SYBR Green Master Mix (applied Biosystems) technology. The levels of target gene expression were normalized to GAPDH expression levels.

Primers used for qRT-PCR were as follows:

**IL-12 p35**
F: 5’-GGCATCCAGCAGCTCCTCTC-3’,
R: 5’-ACCCCTGGCCAAACTGAGGTG-3’;

**IL-12 p40**
F: 5’-TGGTTTGGCCATCGTTTGTGCTG-3’,
R: 5’-ACAGGTGAGGTTCACTGTSSCT-3’;

**IL-6**
F: 5’-GAGGATACCACTCCCAACAGA-3’,
R: 5’-AAGTGCATCATCGTTGTTCAT -3’;

**IL-10**
F: 5’-GGACTGCCTTCAGCCAGGTG-3’,
R: 5’-AGAAATCGATGACAGCGCCTC-3’;

**Stx6**
F: 5’-GGAGAGTTACAGAAAGCAGTCA-3’,
R: 5’-CCCTGAAGGAGCTCTGTCCAT-3’;

**VAMP3**
F: 5’-TGCTGCAAGTTGAAGAGAAAG-3’,
R: 5’-TGATCCCTATCGCCCACATC-3’;

**VAMP7**
F: 5’-ACCTTCGCCCTCAGTCAAT-3’,
R: 5’-GGCAAGGATAGTGTTCC-3’;

**Vit-1B**
F: 5’-TACCGGAAGGACCTTGCTAAAC-3’,
R: 5’-TCCAGGAGCGGCTGTCA-3’;
**Rab27a**

F: 5’-TGCAGTTATGGGACACGGCG-3’,  
R: 5’-GGGGATTCCGTACTTCTCGGC-3’;

**GAPDH**

F: 5’-AGAAGGTGGTGAGCAGGCATC-3’,  
R: 5’-AGAAGGTGGAAGAGTGGGAGTTG-3’.

**Western blot**

In silencing experiments BM-cDCs were collected 48 hrs post-transfection. In experiments with 3LL, 2x10^5 DCs were isolated from spleens by microbeads isolation, or from lungs by FACS sorting.

Cells were washed and lysed with NP-40 plus protease inhibitors. The lysates were centrifuged at 13,000g for 10 min at 4 ℃. The supernatants were boiled for 10 min and separated by SDS-PAGE using 12% polyacrylamide gel. Immunoblotting was performed to quantify SNARE proteins. Antibodies used: mouse α-Rab27a (a gift of M. Seabra, Imperial College London, UK), rabbit α-VAMP3 and mouse α-VAMP7 (a kind gift of A. Peden, University of Sheffield, Sheffield, UK), mouse α-γtubulin (Sigma) and goat α-mouse-HRP (Pierce).

**ELISA**

Cytokine secretion after CpG B stimulation in cell culture supernatants was assessed by ELISA assay, according to manufacturer’s protocols. IL-12 (p70), IL-12 (p40) and IL-10 were measured with Ready-Set-Go! ELISA kit purchased from eBioscience; IL-6 was measured with ELISA MAX kit from Biolegend; IFN-γ was measured with ELISA antibodies from BD Pharmigen.

**Expression of GFP-constructs**

GFP plasmids and mRNA sequences were transfected with Amaxa nucleofector according to the manufacturer’s instructions. BM-DCs transfected with GFP plasmids were collected after 24 hrs, whereas cells transfected with mRNA were used for experiments 5 hrs later.
**Imaging**

To induce maturation, 1-2 x10^5 DCs were stimulated with the TLR9 agonist CpG B (10 µg/ml) for 3-4 hours. For synapse experiments, DCs were stimulated and pulsed with 10nM MHC class I restricted peptide of OVA 257-264 (SIINFEKL) and transferred to slides coated with fibronectin (Sigma-Aldrich; 10ug/ml). OT-I cells were added to DCs in a 1:1 ratio and incubated at 37°C for 30 minutes. Non-adherent T cells were removed by washing the slides with PBS several times. Cells were fixed (4% paraformaldehyde), permeabilized (PBS/BSA 0,2%/saponin 0,05%), and immunolabeled. The following antibodies were used: rat anti-IL-12 p40/p70 (BD Pharmigen), mouse anti-VAMP7 and rabbit anti-VAMP3 (gift of A. Peden, University of Sheffield, Sheffield, UK), mouse anti-Syntaxin 6 (BD Transduction Laboratories), rabbit anti-GFP (Molecular Probes), rat anti-LAMP-1 and goat anti-EEA1 (BD Pharmigen), rabbit anti-Giantin (provided by A. Marcello, ICGEB, Trieste, IT), mouse anti-KDEL (Enzo Lifesciences). All secondary Alexa-tagged antibodies were obtained from Molecular Probes.

Confocal images were acquired with a LSM510 META Axiovert 200M reverse microscope with a 63x and a 100x objectives (Carl Zeiss, Inc.).

Z-stack projection of slides, three-dimensional reconstruction and image analysis were performed using Volocity® 3D Image Analysis Software (Perkin Elmer).

**Silencing of VAMP7 and VAMP3**

Non-targeting siRNA were purchased from Thermo Scientific (Dharmacon RNAi Technology) and Invitrogen, and used as control siRNA. ON-TARGET plus siRNA targeting VAMP3 and VAMP7 were purchased by Thermo Scientific (Dharmacon RNAi Technology) and used as specific VAMP3 and VAMP7 siRNA, respectively.

BM-cDCs were transfected with 2 µM of siRNA using the Amaxa Nucleofector according to the manufacturer’s instructions. 8 x 10^6 wt BM-cDCs at day 5 of culture were resuspended in 100 µl mouse DC nucleofection solution, siRNA was added, and the mixed samples were transferred into cuvettes and transfected by using a program specific for immature DCs. Pre-warmed IMDM complete medium supplemented with F13-L was added to each cuvette after transfection to rapidly dilute the reagents and promote cell survival following shock-electroporation. The
transfected cDCs were collected and seeded into 24-well plates containing complete IMDM plus Flt3L. Cells were collected 48 h after transfection for sequent experiments.

**Functional in vitro assays**

BM-cDCs isolated by magnetic beads were washed, resuspended in complete IMDM supplemented with Flt3 ligand, and put in 96-well culture plates for 0, 1.5, 3 and 6 hours at 37°C in the presence of LPS or CpG-B at 1 μg/ml. Cell pellets were lysed to extract RNA for the qRT-PCRs.

Silenced BM-cDCs were collected after 48 hrs, washed, resuspended in complete IMDM supplemented with Flt3L ligand, and cultured in the presence of CpG-B at 1 μg/ml. Culture supernatants were collected after 5 hours for ELISA analysis. Cell pellets were lysed either to extract RNA for the qRT-PCRs and for the SDS-page analysis of the protein levels.

For the *in vitro maturation assay*, silenced BM-cDCs were stimulated with CpG B at 1 μg/ml at 37°C, and up-regulation of CD86 and MHC class II was measured by FACS analysis 5 hrs later.

For the *viability assay* silenced BM-cDCs were stained with the antibodies 7AAD and Annexin V, according to the manufacturer’s instructions, for the FACS analysis.

BM-cDCs co-coltured with 3LL-D122 cell line for 3 days were detached from the traswells and stimulated with 1 μg/ml CpG B. After 6 hrs of stimulation, cell supernatants were collected for the measure of cytokines secretion, whereas pellets were lysed for the mRNA extraction and analysis by rt-PCR.

**T cell priming**

2x10⁴ silenced-BM-cDC, WT and VAMP7 KO BM-cDCs or DCs isolated from control and tumour-bearing mice were pulsed with increasing doses of OVA₃₂₃-₃₃₉ peptide and after 4 hrs washed and plated with purified 10⁴ OT-I. T cell cytokine production (IFN-γ) was measured after 48 hours of co-culture by ELISA.
**Statistical analysis**

All data were reported as the mean ± s.e.m. as calculated using GraphPad Prism 5 software. The unpaired Student’s t test was used to assess significance.
3. Results
3.1. Analysis of SNARE expression in dendritic cells

Based on previous studies in macrophages, we decided to analyze in DCs a set of SNAREs as representatives of different trafficking routes.

We chose: Syntaxin 6, a t-SNARE localized in the trans-Golgi network (TGN) and involved in the trafficking with the endosomes (Murray et al., 2005a; Murray et al., 2005b);

Vti-1B, also necessary for the post-Golgi trafficking of cytokines in macrophages but found as well implicated in late endosome-lysosome fusion and release in other immune cells (Murray et al., 2005b);

VAMP3, a v-SNARE typically present on early and recycling endosomes and involved in the trafficking from TGN to plasma membrane (Manderson et al., 2007; Murray et al., 2005a);

VAMP7, a vesicular SNARE in general associated with late-endosomes, lysosomes and pre-stored granules in different immune cells (Logan et al., 2006; Marcet-Palacios et al., 2008; Ward et al., 2000);

Rab27a, a SNARE-regulator protein correlated to the human Griscelli syndrome, found localized in lysosomes and involved in regulated secretion of cytotoxic granules (Haddad et al., 2001; Menasche et al., 2000).

For our study we used bone marrow-derived DCs (BM-DC) grown for 7 days in complete medium IMDM supplemented with the cytokine Flt3L. Conventional DCs were isolated through B220 microbeads-negative selection.

The first question we asked is whether these SNAREs are regulated during DC maturation. To address this point, BM-DCs were stimulated with two different stimuli: LPS (TLR4 agonist) and CpG B (TLR9 agonist). Cells were collected at 0, 1.5, 3 and 6 hours after stimulation, and gene expression was evaluated by real time PCR.

As shown in Figure 3.1., after 3 to 6 hours of stimulation, CpG B induced a 2-3 fold increase in the mRNA levels of VAMP7 and Rab27a, and a 6 fold increase in Stx6. In contrast, the levels of VAMP3 and Vti-1B remained unchanged (Figure 3.1.A). On the other hand LPS induced only a slight increase in the mRNA levels of Rab27a, and little or no change in VAMP7 and Vti-1B. VAMP3 and Stx6 instead increased 3-4 times upon LPS stimulation (Figure 3.1.B).

These data suggest that the trans-Golgi SNARE Stx6 is upregulated by both agonists, whereas CpG B induces a selective induction in the late endosomal/lysosomal SNARE VAMP7 and Rab27a. In contrast LPS affects preferentially the early endosomal marker VAMP3.
Conventional DCs (cDCs) were differentiated and isolated from the bone marrow of C57 mice, and then stimulated with TLR agonists (CpG B or LPS 1ug/ml) for different times. The total mRNA was isolated from lysed cells and real time PCR was performed. A,B. Bars show mRNA expression (normalized with the housekeeping gene GAPDH) of different SNARE proteins during DC activation with the TLR9 agonist CpG B (A) or with the TLR4 agonist LPS (B), compared with the relative value of expression in not stimulated cDCs. C. Real time PCR showing the expression of p35/IL-12 and p40/IL-12 in cDCs after different times of stimulation with CpG B or LPS. Data are expressed as the mean ± SEM of three independent experiments. *, p<0,05, **, p<0,01, ***,p<0,001.

To correlate the kinetic of SNAREs up-regulation with induction of IL-12 genes we analyzed in parallel p35 and p40 mRNA at early time points after TLRs stimulation (Figure 3.1.C). The limiting subunit of IL-12, p35 is transiently induced by CpG B, with a pick at 3 hours. The p40 subunit is strongly up-regulated by CpG-B (600-fold induction) as early as 3 hrs post stimulation and it is maintained over time. In contrast, LPS stimulation induces only a slight steady increase in p35 and a 100 fold induction in p40 mRNA. Thus, IL12p70 in our cellular system is efficiently induced only by CpG-B with a kinetic that overlaps with the up-regulation of VAMP7, Stx6 and Rab27a.
3.2. Intracellular distribution of SNAREs in DCs

We next analyzed the intracellular distribution of SNAREs in DCs, focusing on those that we found to be regulated by DC maturation. This analysis was performed using antibodies against VAMP3, VAMP7 and Stx6 in DCs stimulated with CpG B for 5 hrs.

VAMP3 distribution was mostly vesicular and it colocalizes with EEA-1 (Figure 3.2.), indicating that in DCs VAMP3 is a marker for early endocytic compartments, in line with what has been previously described in other cellular models (Bajno et al., 2000; Murray et al., 2005a).

![Figure 3.2. VAMP3 localization in the early endosomes]

*IF detection in BM-derived cDCs of VAMP3 (green) and EEA-1 (red). Z-stack images were taken and analyzed by confocal microscopy. 3D images were reconstituted using the software Volocity.*

VAMP7 localization varies depending on the cell type analyzed; it has been found in the endoplasmic reticulum (Siddiqi et al., 2006), in Golgi apparatus (Advani et al., 1999), in late endosomes and in lysosomes (Advani et al., 1999; Martinez-Arca et al., 2003) and secretory granules (Logan et al., 2006; Marcet-Palacios et al., 2008). In absence of its longin domain it can mislocalize also in the early endosomes (Martinez-Arca et al., 2003).

The localization of VAMP7 has not been described in DCs. Therefore we performed immunolabelling to define its intracellular distribution in the endocytic compartments of DCs using markers for different organelles.

The first marker analyzed was KDEL, which defines the endoplasmic reticulum. In this case a good degree of colocalization is detectable between KDEL and VAMP7 (Figure 3.3.A).

The second marker that I considered was Giantin, which stains for the Golgi complex. In this case VAMP7 didn’t show any appreciable degree of colocalization with this marker (Figure 3.3.B).

The third marker analysed was LAMP-1, which mark the late-endosomes and the lysosomes. In this case a very high level of co-localization is present between this marker and VAMP7 (Figure 3.3.C).
We concluded that VAMP7 in stimulated DCs is mostly localized in early biosynthetic compartments and in lysosomes-like compartments.

![Figure 3.3. VAMP7 intracellular localization](image)

*Figure 3.3. VAMP7 intracellular localization*

IF detection in BM-derived cDCs of VAMP7 (green) and (A) KDEL (red), (B) Giantin (red) and (C) LAMP-1 (red). IF Z-stack images were taken and analyzed by confocal microscopy. 3D images were reconstituted using the software Volocity. Insets represent magnifications of parts of the cells.

Stx6 localization was assumed from the literature, being this SNARE considered a marker of the TGN (Manderson et al., 2007; Murray et al., 2005a).

### 3.3. VAMP3 and VAMP7 polarize during the formation of the immunological synapse

We next evaluated whether synapse formation induces relocalization of SNAREs in DCs. As a model system we used BM-DCs and OVA specific CD8$^+$ T cells (OT-I). DCs were stimulated for 3hrs with CpG-B and loaded or not with OVA class-I peptide, washed and mixed with OT-I cells for 30 minutes to induce synapse formation. After washing off non-adherent cells, slides were fixed and analyzed by confocal microscopy.

To visualize endogenous SNAREs we performed immunolabelling with antibodies against Syntaxin 6, VAMP-3 and VAMP-7.
Results

Figure 3.4. SNAREs differential intracellular recruitment at the immunological synapse
BM-DCs were activated by CpG-B, loaded or not loaded with peptide, mixed for 30 minutes with OVA class I-specific T cells and stained with VAMP7 antibody. The panels show representative images of VAMP3(A), VAMP7(B) and Syntaxin 6(C), differentially polarized towards the T cell in DCs loaded or not with the antigen. For analysis, SNAREs were scored as polarized when the ratio between the MFI of plasma membrane in contact with the T cell and the MFI of the total DC plasma membrane was >1. Plotted data show the percentages of SNAREs polarized in conjugates with T and DCs loaded or not with Ag. Confocal Z-stack images with at least 30 conjugates were analyzed for each condition.

We quantified for each protein the % of DCs with polarized SNARE in antigen specific synapses versus not specific synapses (DCs not pulsed with OVA peptides). SNAREs were scored as polarized when the ratio between the mean fluorescence intensity (MFI) of plasma membrane in contact with the T cell and the MFI of the total DC plasma membrane was >1.

Stx6 didn’t show any antigen specific recruitment as the percentage of polarized cells was similar in Ag specific and not specific synapses (from 35% to 52.6%) (Figure 3.4.C).

In contrast, both vesicular SNAREs showed a high degree of recruitment at the DC-T antigen specific contact region. VAMP3 recruitment increased from 34.7% to 70%, (Figure 3.4.A), whereas the 34.8% of VAMP7 polarized in Ag not specific synapses was elevated to 75% in Ag specific synapses (Figure 3.4.B).

From inspection of our slides and in agreement with previous reports (Huse et al., 2006; Pattu et al., 2012), we observed expression and polarization of SNAREs also on the T cell side of the IS.

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Results

Since it is difficult to discriminate the source (DC or T cell) of SNARE protein in the tight synaptic region, labelling endogenous SNAREs may lead to misinterpretation.

To overcome this problem we moved to study polarization of exogenously transfected SNARE proteins. We obtained from our collaborator Jennifer Stow (University of Maryland, Brisbane) the plasmids encoding for GFP-tagged VAMP3, VAMP-7 and Syntaxin-6. We could achieve a good efficiency of transfection with low toxicity (79% of viability after 15 hrs), in primary DCs by Amaxa nucleofection of in vitro transcribed mRNA encoding the different SNAREs fusion proteins.

In preliminary experiments we established that the expression of in vitro transcribed mRNA peaks at 5-6 hrs post transfection (Figure 3.5.).

Figure 3.5. Kinetic of GFP expression after transfection of SNARE constructs
BM-DCs grown in medium supplemented with GM-CSF were collected at day 6 and transfected by Amaxa Nucleofector with in vitro-transcribed mRNA of GFP-tagged constructs of syntaxin6, VAMP3 and VAMP7. Panel show GFP expression and relative percentage of positive events in not transfected cells and in DCs 3, 6 and 15 hours post transfection.

Thus cells were stimulated with CpG-B and loaded or not with OVA peptide immediately after transfection. DCs were collected after 5 hrs and mixed with OT-I cells to induce synapse formation.

Consistently with data obtained by labelling endogenous SNAREs we found little recruitment in the case of GFP-Syntaxin 6, that showed a small increase in Ag-specific synapses as compared to Ag-not specific ones, shifting from 36% in the DCs not loaded with the antigen, to 54.2% in antigen-specific synapses (Figure 3.6.C).

Instead, antigen specific polarization was evident for VAMP3 and VAMP7, increasing respectively from 40% for not specific synapses to 76.47% for antigen-specific synapses in the case of VAMP3 (Figure 3.6.A) and from 37.5% to 80% in the case of VAMP7 (Figure 3.6.B).

Thus, we confirmed using two different models that the two vesicular SNAREs VAMP3 and VAMP7 in DCs are recruited at the contact zone with CD8+ T cells in Ag specific synapses,
whereas relocalization of the trans-Golgi protein Stx6 occurs randomly and independently of antigen specificity.

**Figure 3.6. GFP-SNARE recruitment at the IS in DCs**
DCs overexpressing GFP-tagged version of different SNAREs were stimulated with CpG B, loaded or not with peptide and mixed for 30 minutes with OVA class I-specific T cells. Representative confocal planes of GFP-VAMP3 (A), -VAMP7 (B), and -Syntaxin6 (C) and the corresponding quantification of SNARE polarization at the IS in DCs loaded or not with the antigen. (≥30 conjugates/ conditions were analyzed).
3.3. Analysis of co-localization between SNAREs and IL-12

We have previously shown that IL-12 containing vesicles become enriched at the IS contact site in antigen specific IS (Pulecio et al., 2010).

Having established that VAMP3 and VAMP7 become recruited at the IS we next investigated the localization of Stx6, VAMP3 and VAMP7 with respect to intracellular IL-12, at first in DCs not engaged in synapse.

This first analysis was performed using DCs transiently transfected with GFP-tagged SNAREs. Cells were stimulated with CpG-B immediately after transfection, harvested 5 hrs later, fixed and labelled with antibodies against IL-12 for confocal analysis. The colocalization index (Pearson’s coefficient) of the single GFP-SNAREs with IL-12 in DCs was measured on 3D reconstructions of confocal Z-stacks using the Volocity software.

![Figure 3.7. SNAREs colocalize differentially with IL-12](image)

3D reconstruction of confocal z-stacks showing the relative distribution of GFP-SNAREs and IL-12. The insets show the colocalization between the different GFP-SNAREs and IL-12 in proximity of the plasma membrane. PC indicates the relative value of Pearson’s Coefficient.

All the three SNAREs were found enriched with IL-12 in the Golgi area (a station in the biosynthetic pathway of all proteins analyzed), reflecting an accumulation of newly synthesised proteins.

Interestingly, in post-Golgi compartments and in proximity of the plasma membrane IL-12 showed a consistent degree of colocalization only with the late endosomal SNARE VAMP7 whereas Stx6 and VAMP3 had a very low Pearson’s coefficient in any region other than Golgi (Figure 3.7.).

Based on this data we focused our subsequent analysis on the SNARE VAMP7.
To further confirm a preferential localization of intracellular IL-12 with the SNARE VAMP7 and to avoid possible artefacts derived from overexpression of GFP-VAMP7 we next used antibodies against VAMP7 to label the endogenous protein.

As shown in Figure 3.8.A both IL-12 and VAMP7 were highly enriched in the Golgi perinuclear area where we observed strong colocalization between the two proteins, similarly to what has been observed using the overexpressed GFP-VAMP7. Most importantly we could observe colocalization in individual vesicles underneath the cell membrane, with a Pearson’s coefficient of 0.31 (Figure 3.8.A, inset).

**Figure 3.8. VAMP7 colocalizes with the two subunits of IL-12**

*Panel A* Stimulated DCs were labelled with α-p40 (red) and α-VAMP7(green) antibodies. *Panel B* DCs were transfected with a GFP-tagged construct of the p35 subunit of IL-12, and labelled with α-GFP (green) and α-VAMP7 (red) antibodies. The inset shows the colocalization between the two proteins at the plasma membrane. Panels are 3D reconstructions of confocal Z-stack images. The Inset shows partial colocalization between the two proteins at the plasma membrane with their relative P.C.

A major limit of this analysis is that the antibody against IL-12 recognizes only the p40 subunit.

As p40 may exist as a homodimer or associated to p19 to form IL-23, we next sought a way to detect specifically the p35 subunit of IL-12. We tested various commercially available antibodies against IL12/p35 but none gave a clearly detectable signal.

Thus we generated a construct in which the sequence of a recombinant mouse p35 was cloned at the N-terminus, separated by a linker, of GFP sequence into a pcDNA3.1 vector (Figure 3.9.).

Transfection of the plasmid with the Amaxa nucleofector in DCs caused massive cell death.

To overcome this problem we sorted live GFP positive cells and we layered them on coverslips. Cells were labelled with antibodies against VAMP7 and analyzed.
Results

Figure 3.9. Cloning of mouse p35 sequence into a GFP-coding plasmid
Murine p35 open reading frame (upper sequence) was cut from its expression vector. A PCR was performed in order to add an Nhel restriction site at the N-terminal and a BamHI restriction site to the C-terminal replacing the Nhel. The fragment cut with Nhel and BamHI enzymes was then cloned into a pcDNA3.1+ vector containing a GFP sequence, that in this new constructs places at the C-terminus of p35.

As for p40, both proteins were highly enriched and overlapped extensively in the Golgi area (Figure 3.8.B). Interestingly we observed a high co-localization index (P.C.:0.61) in vesicles distributed at the cell periphery (Figure 3.8.B, inset).

This data indicate a selective participation of VAMP7 in the trafficking of the bioactive form of IL-12.

Next we formed synapses with DCs expressing p35-GFP or endogenous p40 and labelled them with antibodies against VAMP7 to visualize recruitment and co-localization at the DC-T interface. To this aim DCs were pulsed with OVA class-I peptide, stimulated with CpG B, and mixed with OT-I cells following the procedure as in 3.2. section.

Figure 3.10. VAMP7 colocalizes with the two subunits of IL-12 at the IS
A) DCs stimulated with CpG B and loaded with Ag were mixed for 30 minutes with antigen specific T cells, fixed and stained with α-VAMP7 (green) and α-p40/p70 (red) antibodies. B) DCs transfected with a GFP-tagged construct of the p35 subunit of IL-12 were stimulated with CpG B, loaded with Ag and mixed for 30 minutes with antigen specific T cells. After washing, cells were fixed and stained with α-GFP (green) and α-VAMP7 (red) antibodies. Panels show 3D reconstructions of confocal Z-stacks of DC-T synapses. The insets show zooms of the DC-T contact sites. Double positive secretory vesicles are indicated by the arrows.
Results

As shown in Figure 3.10., we observed a strong co-polarization of both IL-12/p40 and VAMP7 in the synaptic region. Acquisition of high magnification confocal planes along the synaptic area revealed several dots of co-localization between the p40 and VAMP7 signals (Figure 3.10.A). Of note, extensive overlay with VAMP7 vesicles at the IS was found also in the case of p35-GFP (Figure 3.10.B).

Together these data demonstrate that the bioactive form of IL-12 is found associated to VAMP7 vesicles at the DC-T interface of antigen specific synapses, strongly suggesting that VAMP7 may be the SNARE implicated in the latest stages of transport and exocytosis of the cytokine.

3.4. Depletion of VAMP7 in DCs affects cytokine secretion

Based on this initial characterization we next sought to directly assess the role of VAMP7 in the secretion of IL-12 by performing functional analyses.

The first approach was to deplete VAMP7 by silencing its gene expression with siRNA.

3.4.1. Setting up the method

Silencing in DCs is demanding, therefore we performed a set of preliminary experiments to establish the best working conditions that we will summarize in this section.

Figure 3.11. Different methods and efficiencies for siRNA transfection in DCs

DCs were transfected with a siRNA targeted against VAMP7 (siVAMP7) or with an unrelated siRNA (siIrrelevant) utilizing DOTAP, Lipofectamine and Amaxa nucleofection. The values represent the percentage of protein expression, assessed by western blot analysis, of the VAMP7-depleted cells lysates normalized to the expression in DCs transfected with the unrelated siRNA, 48h after transfection.

We first compared two transfection methods, i.e., lipid-based delivery (with DOTAP and Lipofectamine) and nucleofection (Amaxa). 48 hrs post transfection, cells were collected and lysed for the western blot analysis. Results summarized in Figure 3.11. showed that nucleofection using Amaxa is by far more efficient then lipid-based delivery of siRNA.
However, the disadvantage of this method is the high toxicity. We put a big effort to determine the parameters that affect vitality after transfection and we finally succeeded to establish the conditions to perform functional experiments.

Next we performed tests to identify the best siRNA combination (VAMP7 specific and unrelated) to be used in functional tests. To choose the most appropriate control siRNA we transfected 6 irrelevant siRNA (among those commercially available) to search for the one that does not affect IL-12 secretion with respect to mock transfection. After 48 hrs cells were collected, stimulated with CpG B for 5 hrs and cytokine secretion was evaluated in the different irrelevant siRNA-transfected cells with respect to mock.

As shown in Figure 3.12.A, the secretion in cells transfected with siRNA #1 was identical to that of mock transfected DCs. #3 showed a slight increase whereas #2, #4, #5 and #6 showed a quite consistent reduction. These data indicate that the unrelated siRNA #1 is the most appropriate construct to use as control.

Next we tested different sequences to silence VAMP7 expression in DCs. We transfected 4 siRNA, taking as control the unrelated siRNA #1 chosen in the previous experiment. 48 hrs post-transfection cells were lysed and the protein level of VAMP7 was assessed by western blot. Three out of four siRNA tested showed strong reduction of VAMP7 protein levels (Figure 3.12.B). Oligos #4 yielded the most reproducible efficacy of silencing over several tests.

Figure 3.12. Test on different irrelevant- and VAMP7-targeting siRNA
DCs were transfected with Amaxa nucleofector, using different commercial siRNA targeted against VAMP7 (siVAMP7) or with different unrelated siRNA (siIrrelevant). After 48 hours cells were collected for the functional analysis and for the test of efficiency. A) DCs transfected with mock or with 6 different control siRNA (siIrrelevant) were stimulated for 5 hrs with CpG B, and the levels of p70/IL-12 were measured by ELISA. The values indicate the mean fluorescence intensities read at the specrofotometer. B) DCs transfected with different sequences of siRNA against VAMP7 or with siIrrelevant #1 were lysed and the level of VAMP7 protein was assessed by western blot.
On the base of these data, we selected oligo #4 targeting VAMP7 in combination with control #1 irrelevant siRNA as the most appropriate combination for subsequent experiments.

As vitality post transfection is a primary issue we wanted to verify the viability of DCs 48 hrs after transfection with the selected combinations of oligos against VAMP7 and irrelevant.

**Figure 3.13. VAMP7 depletion in DCs doesn’t affect DC viability or maturation state**
DCs were transfected with siRNA control and VAMP7 and after 48h were collected for the experiments. **A.** Control (silIrrelevant) and VAMP7-silenced (siVAMP7) DCs were stained with Annexin V and 7-AAD to check cell viability in the two different conditions. Dot plots show representative FACS images of 3 independent experiments. **B.** DCs transfected with the two different siRNA (Irrelevant and VAMP7) were stimulated with CpG B for 6 hrs and stained with the maturation markers-antibodies I A<sup>4</sup> (MHC II) and B7 (CD86). Histograms show representative FACS images of 3 independent experiments.

DCs were collected and labelled with 7-AAD and Annexin V, and analysed at FACS. Percentage of apoptotic and necrotic cells was similar in both DCs transfected with VAMP7-targeting siRNA and DCs transfected with the irrelevant oligos (*Figure 3.13.A*).

Next, we wanted to compare the maturation profile of cells transfected with VAMP7 and irrelevant siRNA. DCs were collected 48 hrs post transfection, stimulated with CpG B for 6 hrs and stained with antibodies against the maturation markers MHC class II and CD86.

As shown in *Figure 3.13.B*, VAMP7 and irrelevant-transfected DCs express the same maturation profile.

These data indicate that DCs transfected with the selected combination of control and VAMP7-targeting siRNA show comparable vitality and maturation capacity.
3.4.2. Cytokine secretion

After setting up the best working conditions, we moved to test the effects of the depletion of VAMP7 on cytokine secretion.

Cells transfected with siRNA #4 against VAMP7 or with the unrelated siRNA control #1 were collected after 48 hours and stimulated with CpG B. After the stimulation the supernatants were collected to perform series of ELISA assays in order to check the concentration of secreted cytokines in VAMP7-depleted DCs compared to the controls.

As shown in Figure 3.14, we could observe a significant and reproducible decrease (-24,1±2,2%) in the concentration of secreted p70/IL-12 protein.

In contrast the amount of the IL-12 p40 subunit was very modestly affected by VAMP7 depletion (-4,6±1,3%).

![Figure 3.14. VAMP7 depletion in DCs selectively impairs IL-12 secretion in DCs](image)

DCs were transfected with a siRNA targeted against VAMP7 (siVAMP7) or with an unrelated siRNA (siIrrelevant). Control and VAMP7-depleted cells were stimulated with CpG B for 5 hrs. The relative content of p70, p40, IL-6 and IL-10 secreted in cell culture supernatants was determined by ELISA assay. Bars show means ± SEM of four independent experiments.

A slight decrease (-12,7±2,4%) was present also in the levels of another inflammatory cytokine, IL-6, which indicates a selective but not exclusive implication of VAMP7 in the secretion of bioactive IL-12. Of note, secretion of the inhibitory cytokine IL-10 (+5,5±3,4%) was slightly increased in VAMP7 depleted cells (Figure 3.14.).

To fully establish that decreased levels of IL-12/p70 observed in the supernatant are due to reduced secretion, we measured p35 and IL-6 mRNA in control and silenced cells stimulated for 3 hours with CpG B. No statistically significant decrease was observed for p35 nor for IL-6 mRNA in VAMP7-silenced cells (Figure 3.15.).

Thus we conclude that silencing VAMP7 selectively affects protein secretion rather than gene induction.
Results

Figure 3.15. VAMP7 depletion in DCs doesn’t affect the transcription of IL-12 and IL-6 in DCs.
DCs were transfected with a siRNA targeted against VAMP7 (siVAMP7) or with an unrelated siRNA (siIrrelevant). After 48 hrs DCs were collected and stimulated with CpG B for 3 hours. The relative quantity of p35 and IL-6 mRNA in cell lysates was determined by RT-PCR. Bars show means ± SEM of four independent experiments.

3.4.3. T-cell priming

Having established a role for VAMP7 in the secretion of bioactive IL-12, we next examined whether the impairment caused by the silencing of this SNARE may have an impact on the ability of DCs to prime naïve T cells.

To test this hypothesis we performed T cell priming assays using control and VAMP7-silenced DCs. Cells were stimulated with CpG B and pulsed with different concentrations of the peptide OVA class I. After washing out peptide and stimulus, CD8+ purified naive OT-I cells were added to the DCs containing wells and the cell co-culture supernatants were harvested after 48 hrs for IFN-γ content determination.

Figure 3.16. VAMP7 depletion in DCs affects the T cell priming capacity in DCs.
Control (siIrrelevant) and VAMP7-silenced (siVAMP7) cDCs (2·10^4/condition) were stimulated with CpG B (10 µg/ml) and different doses of OVA class I peptide for 4 hours, then washed to remove the supernatants and mixed with 10^5 OVA-specific T cells. 48 hours cell culture supernatants were collected and IFN-γ secretion was measured by ELISA assay. Values are means ± SEM of relative secretion of 4 independent experiments. (*, p<0.05).

The levels of IFN-γ secreted by OT-I cells after 48hrs were slightly but reproducibly decreased in co-cultures with VAMP7-depleted DCs (Figure 3.16.).
We conclude that impaired IL-12 secretion in VAMP7-depleted cells interferes with the acquisition of effector functions in T cells.

3.5. IL-12 secretion and T cell priming in VAMP7 knock-out DCs

So far our results have highlighted the role of VAMP7 in DCs using acute depletion of the protein by siRNA. Next we decided to confirm and extend these results in a model of constitutive VAMP7 depletion.

For this purpose we used a model of bone marrow-derived DCs from VAMP7 knock out (KO) mice. These mice develop normally up to adulthood, and are viable and fertile. Elimination of TI-VAMP does not lead to any major developmental deficiencies; the main defects identified in TI-VAMP KO mice are decreased body temperature, increased third ventricle volume, blood glycerol and anxiety. We have obtained the bone marrow of VAMP-7 knockout animals by our collaborator T. Galli (Institut J.Monod, Paris, FR).

We differentiated DCs from the bone–marrow of VAMP7 KO mice or wild type (WT) littermates. Differentiation into CD11c+ cells proceeded equally between the two strains, and expression of the maturation marker CD86 was similar in wt and ko DCs, indicating that VAMP7 does not interfere with DC development (Figure 3.17.).

Figure 3.17. VAMP7 KO DCs have a normal development in terms of differentiation and maturation. BM-DCs from wt and VAMP7 ko mice were collected after 7 days of culture and stained for FACS analysis. A) WT and VAMP7-KO DCs were stained with antibodies against CD11c and analysed at FACS to check cell differentiation. B) DCs were stained with the maturation marker-antibody B7 (CD86). Histograms show expression of the marker in WT (blue line) and in KO DCs (green line).

3.5.1. Cytokine secretion

To test their function, BM-DCs derived from VAMP7 KO mice and from their wild type littermates (WT) were isolated by negative selection with B220 microbeads to obtain
conventional DCs. Cells were stimulated for 3, 6 and 15 hrs with CpG B and cytokine secretion was measured by ELISA.

As shown in Figure 3.18, the amount of p70/IL-12 secreted by VAMP-7 KO cells was significantly decreased as compared to wt cells, at all time points tested. This decrease was specific for bioactive IL-12, as the amount of p40, IL-6 and IL-10 was similar between the two genotypes. These data are in agreement with our experiments with siRNA depleted cells, strongly supporting that VAMP7 expression in DCs is selectively involved in secretion of bioactive IL-12.

3.5.2. T-cell priming

Having confirmed that VAMP7 KO DCs show impaired IL12p70 secretion, we next tested their capacity to prime T cells.

To this aim we performed the same priming assay described for siRNA VAMP7-depleted DCs in section 3.4.3. Cells were stimulated with CpG-B, loaded with peptide and mixed with CD8⁺ OT-I cells. The concentration of IFN-γ secreted by activated T cells was measured in the cell culture supernatants after 48 hours of interaction. We observed less amount of IFN-γ secreted by T cells in co-culture with VAMP7 KO DCs as compared to WT (Figure 3.19.) thus confirming the results obtained with VAMP7-silenced DCs.
Results

Figure 3.19. VAMP7 KO DCs have impaired T cell priming abilities
Control (WT) and VAMP7 knock out (KO) cDCs (2·10^4/condition) were stimulated with CpG B (10 µg/ml) and different doses of OVA class I peptide for 4 hours, then washed to remove the supernatants and mixed with 10^5 OVA-specific T cells. 48 hours cell culture supernatants were collected and IFN-γ secretion was measured by ELISA. The graph shows the data from a representative experiment.

3.6. VAMP3 is required for TLR9 signalling

In parallel experiments we continued to test the function of the vesicular SNARE VAMP3, even if initial co-localization studies indicated poor overlap with IL-12 containing vesicles. We will briefly describe the preliminary results of these experiments in this section.

We performed siRNA studies to analyze the effect of VAMP3 depletion. As described for VAMP7 we first selected the most appropriate combination of siRNA. DCs were transfected with Amaxa nucleofector, using 3 different VAMP3 specific siRNA oligos and two different unrelated siRNA. After 48 hrs cells were collected and lysed for western blot analysis of VAMP3 protein level. All the 3 targeting siRNA showed a robust inhibition of VAMP3 protein expression level (Figure 3.20.). Thus we chose the irrelevant #1 (already used as control in VAMP7-silencing experiments) and the VAMP3-targeting siRNA #1 as combination for the subsequent experiments.

Figure 3.20. Test on different irrelevant- and VAMP3-targeting siRNA
DCs were transfected with Amaxa nucleofector, using different commercial siRNA targeted against VAMP3 (siVAMP3) or with different unrelated siRNA (siIrrelevant). After 48 hours cells were collected, lysed and the level of VAMP3 protein was assessed by western blot.
As done for cells transfected with the combination of siRNA control and VAMP7-targeted, we next analyzed the vitality of DCs 48 hrs after transfection of the selected control and VAMP3 siRNA. Cells were collected and stained with a marker for vitality and analysed at FACS. The effect on cell viability was similar for irrelevant #1 or VAMP3 #1. Subsequently, we compared the maturation profile of cells transfected with VAMP3 and irrelevant siRNA. DCs were collected 48 hrs post transfection, stimulated with CpG B for 6 hrs and stained with antibodies against the maturation markers MHC class II and CD86. FACS analysis showed no significant differences of MHC II and CD86 expression in VAMP3-depleted cells compared to the irrelevant-transfected DCs. These data indicated that DCs transfected with the selected combination of control and VAMP3-targeting siRNA show comparable vitality and maturation capacity.

After setting up the optimal working conditions, we wanted to study the effects of the depletion of VAMP3 on cytokine secretion. Cells transfected with siRNA #1 against VAMP3 or with the unrelated siRNA control #1 were collected after 48 hrs and stimulated with CpG B for 5 hrs. After stimulation the supernatants were collected to perform cytokine secretion analysis by ELISA.

![Figure 3.21. VAMP3 depletion impairs cytokine secretion in DCs](image)

*DCs were transfected with a siRNA targeted against VAMP3 (siVAMP3) or with an unrelated siRNA (siIrrelevant). Control and VAMP3-depleted cells were stimulated with CpG B for 5 hrs. The relative content of p70, p40 and IL-6 secreted in cell culture supernatants was determined by ELISA assay. Bars show means ± SEM of four independent experiments.*

A significant decrease was observed for all the cytokines analyzed (-42.2±2.7% for p70/IL-12, -24.22±8.5% for p40, -40.49±7.1% for IL-6), indicating a strong inhibition in the secretion of inflammatory cytokines in VAMP3-depleted cells (*Figure 3.21.*).
Results

Figure 3.22. VAMP3 depletion affects cytokine transcription in DCs.
DCs were transfected with a siRNA targeted against VAMP3 (siVAMP3) or with an unrelated siRNA (siIrrelevant). After 48 hrs DCs were collected and stimulated with CpG B for 3 hours. The relative quantity of p35, p40 and IL-6 mRNA in cell lysates was determined by RT-PCR. Bars show the data of a representative experiment.

The significant inhibition of all inflammatory cytokines in VAMP-3 depleted cells suggests an effect on induction of the TLR9 pathway. To address this we measured by real time PCR the levels of p35, p40 and IL-6 mRNA in control and silenced cells stimulated for 3 hours with CpG B. As shown in Figure 3.22., p35, p40 and IL-6 transcripts were strongly reduced in VAMP3-silenced cells after CpG-B stimulation as compared to control. Therefore these data suggest that VAMP-3 plays a role in TLR9 upstream signalling events, which will be further investigated.
3.7. DCs and cancer

During the last years very large efforts have been made in the field of cancer immunology in order to understand the events occurring during the establishment of a tumour microenvironment and how to revert the state of immune suppression that follows this establishment.

One of the most important mediators which has been identified for its strong anti-tumour activity in mouse tumour models is IL-12. Unfortunately, the factors present in the tumour microenvironment lead to a dramatic shut off of cytokine production in the cells producing this important pro-inflammatory cytokine, such as macrophages and dendritic cell.

At present little is known on the mechanism that account for the block in cytokine secretion observed in tumour exposed DCs. In particular whether trafficking events may be targeted by immunosuppressive mechanism has not been investigated.

Having gain basic knowledge on the biology of SNAREs in cytokine secretion in DCs I dedicated the second part of my experimental work to analyze the modifications taking place in this class of proteins in tumour-educated DCs.

3.7.1. Setting up of an in vitro model of cancer-mediated immunosuppression: DC–3LL co-cultures

The suppression of the innate and adaptive arms of the immune response has been studied using various cancer models. In the context of lung cancer the mechanism of suppression of DCs functions have just begun to be investigated. It has been shown that lung cancer cells induce a block in inflammatory cytokine secretion by DCs and that tumour-educated DCs inhibit T cell proliferation via arginase-1 (Liu et al., 2009). Therefore, based on this published evidences we set-up ex-vivo co-culture of BM-DC with a widely use lung cancer cell line, the highly metastatic 3LL-D122 (Lewis Lung carcinoma). We used transwells to keep the two cell types physically separated and allow recovery of pure DCs afterward. In the lower chamber we seeded 3LL and let them to form a monolayer. DCs that had been differentiated for 6 days in Flt3L were added to the upper transwell chamber. Control DCs were kept into transwell chambers with no 3LL on the bottom of the well. After 3 days of co-cultures, DCs were collected from the transwell for the analysis of cytokine secretion and SNARE expression.
**Cytokine secretion and SNARE expression analysis**

We first examined the levels of the inflammatory and inhibitory cytokines IL-12 and IL-10 in tumour-conditioned DCs. Ctrl and 3LL-DCs were collected after 3 days of co-culture and stimulated with CpG B for 6 hrs. After stimulation cell supernatants were collected and concentrations of secreted cytokines were analysed by ELISA.

As shown in Figure 3.23, at steady state both cytokines had the same levels in ctrl and 3LL-DCs. No induction was observed in IL-10 levels that remained comparable even after CpG-B stimulation in both conditions. In contrast, a very strong decrease in the levels of IL-12/p70 induced by CpG-B was observed in 3LL-DC supernatants compared to the control. These data indicate that 3LL-secreted factors impair the ability of secreting the pro-inflammatory cytokine IL-12 in DCs.

Having observed an impairment in cytokine secretion, we wanted to look at the SNARE pattern in DCs co-coltured with 3LL.

DCs were collected from the transwells, lysed, and mRNA levels of different SNAREs was analysed by real time PCR in tumour-conditioned and control DCs.

![Figure 3.23. Cytokine secretion of BM-DCs co-coltured with 3LL-D122](image)

**Figure 3.23. Cytokine secretion of BM-DCs co-coltured with 3LL-D122**

BM-DCs were co-cultured into a transwell system for 3 days. Control DCs were put in transwells with only medium at the bottom of the well, whereas the tumour-conditioned DCs were put in transwells with a 3LL-D122 monolayer on the well bottom. 3×10^5 control (CTRL) and tumour-conditioned DCs (3LL) were stimulated (CpG B) or not stimulated (n.s.) with 1µg/ml of CpG B for 6 hours. Supernatants were collected and the concentrations of p70/IL-12 and IL-10 were measured by ELISA essay. The values shown are the mean±SEM of 3 independent experiments.

As in section 3.1 we looked at the transcription levels of VAMP3, VAMP7, Rab27a, Vti1b and Syntaxin 6. The data show a very strong decrease of VAMP3. A certain decrease was observed also in VAMP7 mRNA levels. In contrast, Rab27a, Vti-1B and Stx6 remained unchanged (Figure 3.24.). These results indicate that DCs co-coltured with 3LL show a selective decrease in the transcription of the vesicular SNAREs VAMP3 and VAMP7.
3.7.2. Setting up of an in vivo mouse model of cancer-mediated immunosuppression: lung carcinoma

Tumour establishment affects DC functionality and SNARE expression in the spleen

In studies performed in vivo it has been shown that spleen is crucial in the promotion of peripheral tolerance during cancer progression (Cortez-Retamozo et al., 2012; Ugel et al., 2012). After the first observations obtained with the in vitro model, we examined DCs isolated from the spleen of mice bearing a tumour. We used as in section 3.7.1 the 3LL-D122 Lewis Lung carcinoma, described as a poorly immunogenic and highly metastatic model in C57BL/6 mice (Weiss and Ward, 1987). To implant the tumour, 3LL-D122 cells were injected in the caudal vein (1M of cells/mouse). After 14 days, injected and control mice were sacrificed and spleens were collected for further analysis, which included phenotypic FACS analysis and DCs isolation for functional assays.

First we wanted to analyse the DC subtypes in spleens from tumour-bearing mice compared to the controls. Cell suspensions obtained from spleens by mechanical dissection and enzymatic digestion were stained with antibodies against CD11c, CD11b, Gr-1 and CD8α, and analysed at FACS.
Figure 3.25. Characterization of splenic DCs in healthy and tumour-bearing mice
FACS plots showing total cells from spleens collected from healthy (CTRL) or tumour-bearing (3LL) mice stained for the analysis of expression of CD11c, CD11b, CD8 and Gr-1. The gates isolate the CD11c+ DC population in the two conditions. Histograms show the relative expression of Gr-1 in CTRL and 3LL isolated populations A, B and C.

CD11c+ cells percentages in spleens collected from tumour-bearing mice compared with those from control mice were comparable, indicating a similar presence of DCs (Figure 3.25., gate A). DCs in the two conditions were similar also in terms of CD11b and Gr-1 expression, with a low/intermediate expression of CD11b and a low expression of Gr-1 in both cases. Staining with anti CD8α to discriminate the CD8α+ DCs subset that is involved in cross-presentation and IL-12 secretion, did not reveal any significant difference in frequency.

In contrast, increased percentages of CD11c- infiltrating myeloid-derived suppressor cells expressing CD11b+Gr-1int (Figure 3.25., gate B) or CD11b+Gr-1hi (Figure 3.25., gate C) were visible in the tumour-bearing mice. In particular gate B increased Gr-1 expression in tumour-bearing mice (Figure 3.25.).

Together these data indicate that no major alterations are visible in splenic DCs from tumour-bearing mice, that maintain the same subset composition as in control mice. These results indicate also an enrichment of myeloid-derived cells in the spleen of tumour-bearing mice.

To test splenic DCs functions cells were isolated to a purity of 90% from spleen cell suspension by positive selection with CD11c microbeads. After isolation DCs were stimulated with CpG B for 6 hours, and IL-12 concentration was measured in cell supernatants. As shown in Figure 3.26.A, a strong impairment of IL-12 was observed in DCs from tumour-bearing mice after ex vivo stimulation. These data indicate the presence of a systemic DC anergy in mice bearing lung carcinoma.

Yet, the phenomenon was observed only when the primary tumour was in a very advanced stage, indicating that the peripheral immunosuppression is dependent on the tumour progression.
Next I decided to look at the SNAREs expression in splenic DCs from tumour-bearing mice compared to healthy. DCs isolated by CD11c positive selection were lysed and mRNA levels of SNAREs were analyzed as in section 3.7.1. As shown in Figure 3.26.B all the SNAREs analyzed were down-regulated at mRNA level in the splenic DCs of tumour-bearing mice compared with the controls.

**Analysis of DCs in primary tumour-site**

After the analysis of BM-DCs and splenic DCs, we moved to analyze DCs directly infiltrating lung tumours.

Isolating DCs from lungs of tumour-bearing hosts presented some difficulties because of a large amount of viscous material that complicated single cell suspension. We solved the problem by testing several tissue digestion enzymes and by repeated filtration.

Very little is known about DC changes during tumour development in lungs. Thus, after setting up the optimal conditions for single cell isolation, we sought to analyse the phenotype of DCs in control and tumour-lungs. Lungs are organs with a high structural and cellular complexity. A network of myeloid cells, including a large fraction CD11c+ alveolar macrophages are present in the lung. Indeed previous studies of DCs in lung cancer were limited to the analysis of ectopically implanted tumour (3LL cells injected subcutaneously) or were based on isolation of total CD11c lung cells, thus a mixed macrophage/DCs population. To provide a first detailed phenotype of DCs subsets in the lung of tumour bearing animals we performed multicolor FACS analysis according to a procedure described in the context of influenza infection. This includes
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physical parameters and other surface markers such as MHC II and CD103, to better distinguish pulmonary DCs from alveolar and infiltrating macrophages (present in this organ with a phenotype very similar to DCs) and also to discriminate between organ-resident cells from the infiltrated ones (Figure 3.27.).

First, we gated on live hematopoietic CD45\(^+\) cell fraction. From CD45\(^+\) cells we discriminated SSC\(^{\text{low/int}}\)MHCII\(^{\text{hi}}\)CD11c\(^+\) DC (gate B) from SSC\(^{\text{hi}}\)MHCII\(^{\text{int}}\)CD11c\(^+\) macrophages (gate A).

Figure 3.27. Characterization of immune cell populations in healthy and tumour-bearing lungs. FACS plots showing total cells from lungs collected from healthy or tumour-bearing mice stained for the analysis of expression of CD45, CD11c, CD11b, MHC II and CD103. Hematopoietic cells were defined on the base of expression of CD45; MHC II and SSC physical parameter were used to define DCs (gate B) from macrophages (gate A). The CD11c\(^{\text{hi}}\)MHCII\(^{\text{hi}}\) fraction (gate C) was divided on the base of the expression of CD11b (gate D) or CD103 (gate E) markers.

In tumour-bearing lungs we observed a strong enrichment in the gate A, corresponding to cells with an intermediate expression of both MHCII and CD11c, likely representing myeloid-infiltrated cells and that deserve further characterization. From gate B we identified the CD11c\(^{\text{hi}}\)MHCII\(^{\text{hi}}\) DCs (gate C), that were strongly enriched in tumour-bearing lungs. A further observation was done in the composition of “migratory” CD103\(^+\) (gate E) and myeloid CD11b\(^+\) (gate D) DCs in tumour-lungs. We observed an increase in the proportion of CD11b\(^+\) cells and loss of CD103\(^+\) cells. A similar depletion of CD103\(^+\) cells was described in the context of influenza virus infection (Helft et al., 2012). The levels of Gr-1 remained low and we did not observe substantial changes in the intensity of MHC-II staining. Thus, established lung tumours are prevalently infiltrated by CD11b\(^+\) cells DCs. (Figure 3.27.).
Based on this gating strategy we isolated the CD11c⁺MHCII⁺ (gate C in Figure 3.27.) population by FACS sorting, reaching a purity of 95%. After isolation, control and tumour-DCs were stimulated with or not with CpG B for 15 hrs and the concentration of IL-12 and IL-10 in cell supernatants were measured by ELISA.

**Figure 3.28. Cytokine secretion capacity of normal and tumour-associated lung DCs**

DCs were isolated by FACS sorting from the lungs of 6 weeks old C57BL/6 mice previously injected or not injected with 3LL-D122 tumour cells. 2∗10⁵ control (CTRL) and tumour-associated DCs (3LL) were stimulated (CpG B) or not stimulated (n.s.) with 1µg/ml of CpG B for 15 hours. Supernatants were collected and the concentrations of p70/IL-12 and IL-10 were measured by ELISA essay. The graphs shown are representative of 4 independent experiments.

Results indicate a strong impairment in IL-12 secretion of ex vivo stimulated tumour-DCs. On the other hand in DCs from tumour-bearing lungs we observed an increase in the levels of IL-10 secreted both at steady state and upon CpG stimulation (Figure 3.28, Figure 3.). These data indicate a strong differentiation in tumour-DCs towards an immunoregulatory phenotype, and confirmed the results obtained in vitro DC co-cultures experiments and in vivo splenic DCs.

**Figure 3.29. SNARE expression in normal and tumour-associated lung DCs**

DCs were isolated by FACS sorting from control and tumour-bearing lungs. A) mRNA was isolated and measured by RT-PCR. Bars indicate relative mRNA expression (normalized with the housekeeping gene GAPDH) of different SNARE proteins in tumour-DCs (3LL), compared with relative expression in control-DCs (CTRL). Values are means±SE from 5 to 2 independent experiments. B) Protein levels of different SNAREs in control and tumour-DCs were checked by immunoblotting of DC lysates.
Remarkably we also found altered SNARE expression levels in DCs isolated from lung tumours. Transcription levels of VAMP3, Syntaxin 6 and Vti1b were decreased in tumour associated DCs as compared to controls (Figure 3.29.A). VAMP3 and to a certain extend Rab27a showed a consistent decrease also at protein level (Figure 3.29.B).

Together all these data unveil a new mechanism of DC suppression in lung cancer that depends on targeting the expression of trafficking protein.
4. Discussion
Dendritic cells are professional antigen presenting cells able to sense dangerous signals circulating in the host and to transmit them to naive CD8$^+$ T cells thus initiating adaptive immunity. This communication is made possible by the production by DCs of 3 major signals: antigen-loaded MHC complexes (signal 1), expression in the plasma membrane of adhesion and co-stimulatory molecules (signal 2) and secretion of soluble mediators (signal 3).

Among the soluble mediators, the cytokine IL-12 is fundamental for the efficient priming of CD4$^+$ and CD8$^+$ T cells into Th1 and CTLs respectively, both implicated in the induction of cytotoxicity against pathogens, viral infections and cancer cells.

The communication between DCs and T cells takes place into the delimited space of the immunological synapse, where signals 1 and 2 can be paired with the complementary molecules present on T cell surface. About the third signal, we have previously shown that during IS formation, the microtubule organizing centre of the DC becomes polarized towards the contact area with the naive T cell, bringing newly synthesised intracellular vesicles of IL-12 underneath the plasma membrane. This IL-12 polarization triggers a more efficient T cell priming compared to the not polarized DCs in the very early events of the DC-T cell contact, as shown by upregulation of pSTAT4 in the T cells in antigen-specific IS, after 30 minutes of interaction.

Starting from these observations, the first part of this thesis aimed at identifying the intracellular pathway of IL-12 secretion by characterizing the trafficking molecules involved in its vesicular transport to the IS. For this purpose, the identification of the specific SNARE proteins involved in the intracellular transport steps is a very helpful experimental approach for characterizing the trafficking routes used by cytokines to exit the cell. Our results show that the late endosomal SNARE VAMP7 is involved in IL-12 synaptic secretion and in the effective CD8$^+$ T cell priming. Furthermore we identify the early-recycling endosomal VAMP3 as regulator of the DC cytokine production via TLR9 signalling.

In murine bone-marrow derived conventional DCs we observed upregulation of SNARE transcription upon DC maturation; this upregulation was different among SNAREs analysed, and surprisingly showed also a specificity for the TLR engaged (TLR4 upregulates VAMP3, TLR9 upregulates VAMP7 and Stx6). These data indicate the presence of a fine regulation in the expression of trafficking molecules, which depends on the maturation pathway engaged. The diverse pattern of SNARE and cytokine induced by the different stimuli suggests that, in the economy of the cell, soluble mediators and their own specific transporter are co-regulated.
In this respect, Stx6 that control trans-Golgi trafficking, a step common to all secreted proteins, is induced by both stimuli tested. In contrast, VAMP7 and Rab27a are selectively induced by CpG-B, the agonist that triggers efficient release of IL-12p70. Although not conclusive, this observation represents a first initial correlation between bioactive IL-12 and its secretory pathway.

SNAREs have demonstrated a crucial role in intracellular trafficking and release of immune mediators from macrophages and T cells, even if through different routes. SNAREs are also important for the cell polarity at the IS in T cells and during delivery of membrane to the phagocytic cup in APCs. Thus, investigation of the locations of SNAREs in immune cells is important for defining immunological pathways.

A first co-localization analysis using commercial antibodies that uniquely defines intracellular compartments in most cells revealed that the three SNAREs mostly induced by DC maturation - Stx6, VAMP3 and VAMP7-, map quite precisely distinct intracellular compartments of DCs: trans-Golgi network, early endosomes and lysosome-like endosomes respectively.

In the context of the IS, the vesicular VAMP3 and VAMP7 showed an antigen-dependent polarization towards the DC-T cell contact, whereas Stx6 had a random localization: this observation suggests a polarity of the endosomal compartments, but not of the TGN, indicating an active recruitment of the early and late endosomes in the IS region in antigen-specific synapses. We also observed a not specific background of polarization. The percentage of polarization towards the contact region in not antigen specific DC-T contacts was between 35 and 40% for all SNAREs analysed. This indicates that during short antigen not-specific contacts, ligation of surface molecules, most likely adhesion molecules and integrins, is able to induce transient events of polarization of all compartments. Instead, formation of antigen specific synapses stabilizes the polarized flux of post-Golgi trafficking vesicles toward the IS.

An important technical advance in this work is represented by the optimization of the conditions to overexpress GFP-tagged constructs with limited toxicity. In vitro transcribed mRNA transfected with Amaxa nucleofector in GM-CSF differentiated BMDCs assures a very fast protein expression and a 100% recovery. This technique allowed us to firmly establish recruitment of DCs SNARE at the IS, avoiding misinterpretation that may derive from contributing T cell SNARE when labelling the endogenous protein. Moreover, it poses the basis for future time-lapse imaging studies.

In the analysis of localization of IL-12, the cytokine was found accumulated in the Golgi area together with all the SNAREs analysed, indicating that all the proteins considered are synthesised and initially localized in the same compartment. In contrast, this general co-localization is lost when
Departing from the Golgi region and approaching to the plasma membrane. In this case VAMP7 is the only SNARE protein found together with IL-12. More specifically, the preferential zone of co-localization was in the close proximity of the plasma membrane, indicating that the final steps before secretion diverge from those of Stx6 and VAMP3, but are common with those of VAMP7. From the analysis of co-localization of the two single subunits of the cytokine, the IL-12-specific and limiting p35 showed a higher co-localization index with VAMP7 as compared to that of the more abundant and less specific p40, confirming that bioactive IL-12p70 intersects VAMP7 vesicles on its way out.

The preferential association of IL-12 with VAMP7 was further emphasized when DCs were engaged in Ag specific synapses. Analysis of the DC-T contact region revealed an enrichment in IL-12 and VAMP7 double-positive vesicles. These results suggest that VAMP7 may regulate the last fusion events before release of bioactive IL-12 in the IS.

This finding was somehow unexpected, as it suggests a pathway of release of bioactive IL-12 through lysosome-like compartments.

Indeed, in other immune cell types (T cells, NK cells, eosinophils, neutrophils) the lysosomal secretion and particularly VAMP7, have been always associated to regulated exocytosis of pre-stored granules (Logan et al., 2006; Marcet-Palacios et al., 2008; Stinchcombe and Griffiths, 2007). Secretory lysosomes in granulocytes are for instance most often preformed structures storing secretory products while awaiting stimulation, and CTLs produce the protein components of cytotoxic granules and the structures themselves, releasing their contents only upon triggering of the TCR signalling (Blott and Griffiths, 2002).

By analogy with CTLs, the co-localization of newly synthesized IL-12 with lysosome-like compartments suggests an early accumulation of IL-12 containing vesicles during DC maturation, that can be rapidly released upon formation of stable synapses by signals delivered through accessories molecules. This hypothesis is supported by preliminary experiments showing that 15 minutes of antigen-specific DC-T cell contacts are enough to induce a burst in IL-12 release (not shown).

In previous studies a regulated secretion in DCs was already observed during IS formation with NK cells and T cells, for the release of “non-classical” cytokines. CD8+ T lymphocytes for instance induce the exocytosis of IL-1β secretory lysosomes from DCs (Gardella et al., 2001); IL-12 and IL-18 in DCs polarize at the interface between the two cells during interaction with NK cells (Borg et al., 2004; Semino et al., 2005). It has been shown that prolonged contact with CD4+ T cells induce
production of IL-12 (Bertrand et al., 2010; Tourret et al., 2010). All these works reinforce the notion that DCs undergo functional polarization in response to prolonged interaction with other cells. Our experiments add to these previous studies a new concept, i.e., that preformed cytokines derived from early encounter with microbial-derived signals can be rapidly released upon a first contact with an antigen specific naïve T cell.

Silencing of VAMP7 in primary DCs was quite a demanding procedure. Amaxa nucleofection gave good results in terms of efficiency but, as expected, transfection of genetic material into antigen-presenting cells (especially the Flt3L derived cells that we use because of their high capacity to produce IL-12 p70) were associated with cell activation and toxicity. To minimize cell death we set up strict conditions for culturing the cells, by keeping extremely stringent conditions of sterility during cell culture and manipulation (with the use of an exclusively dedicated cell culture room and incubator), and minimum mechanical stress. These conditions allowed a good balance between vitality and efficiency. Due to these difficult and delicate conditions extreme care was placed in choosing the best combination of oligos for silencing, as little differences on toxicity could have given artefacts on the functional analysis performed. Once the couple of appropriated oligos was identified, we measured silencing efficiency by the specific siRNA. In the case of VAMP7 the silencing was highly reproducible leading to reduction of 80% both at mRNA and protein level at 48 hrs post-transfection. Importantly, protein depletion was maintained after stimulation of the cells with CpG-B at 48hrs (not shown), indicating that the siRNA oligo is capable to target also the mRNA transcribed de-novo upon stimulation.

Interestingly in DCs stimulated with CpG B, the 80% depletion of VAMP7 led to a 25% of down-regulation in the IL-12 secreted upon TLR9 activation, without affecting the IL-12 mRNA transcribed in DCs. This relatively minor inhibition was nevertheless highly reproducible, as it was observed over many experiments performed, demonstrating that VAMP7 is required for IL-12p70 release. That most of IL-12p70 can still be released when VAMP7 is reduced has two possible explanations: 1) the pathway is highly efficient and 20% of expressed protein can accomplish the transport, 2) most likely, the presence of redundant trafficking routes assure the release of this important mediator to the plasma membrane. This hypothesis is plausible, as the establishment of multiple surface trafficking pathways involving endosomal compartments and redundancy among SNAREs is common to cells with sophisticated membrane compartment organization such as epithelial cells, adipocytes, and neurons (Borisovska et al., 2005; Farr et al., 2009; Zhao et al., 2009), allowing the cells to maintain their major functions. Moreover, as ELISA to detect IL-12p70 is not extremely sensitive, we need to work at high concentration of TLR9 agonist to be able to
detect the protein. It is likely that VAMP7 function would be better disclosed at low physiological doses of stimulation. The other intriguing possibility suggested by our data is that besides being partially required for general IL-12 p70 release, VAMP7 may serve a selective function in synaptic release. Proving this is an important point that we will address in the immediate future by implementing the T cell-induced release assay that we have established using normal, not transfected cells.

In parallel to the impairment of IL-12 secretion, the silencing of VAMP7 caused a slight decrease also in the secretion of IL-6, which again is not due to decreased gene transcription but rather to defective release. In contrast, secretion of the abundant subunit p40, and of the scarce cytokine IL-10 remained unchanged. These observations indicate on one hand a differential trafficking of cytokines inside the cell, and on the other hand the superimposition of trafficking pathways between groups of cytokines. Indeed we could observe colocalization between IL-6 and VAMP7 in preliminary experiments whereas we failed to detect intracellular IL-10 to draw a conclusion. (not shown). Similar observations were done in macrophages and T cells; in the first case IL-6 and TNF-α follow the same intracellular pathway, yet are released differentially in a multidirectional or in a phagocytic cup-targeted way respectively (Manderson et al., 2007). In the second case synapse-targeted cytokines like IL-10, IL-2 and IFN-γ follow different pathways and are escorted by different SNAREs with respect to multidirectionally delivered cytokines like TNF and IL-4 (Huse et al., 2006).

Downregulation of IL-12 secretion shows repercussions in the capacity of DCs to prime, as indicated by the 30% decrease of IFN-γ secreted by T cells after 2 days of interaction with antigen-pulsed VAMP7-depleted DCs. This is in agreement with previous reports showing that IL-12 is required for survival and acquisition of effector functions in primed CD8+ T cells. Ongoing experiments to exclude interference with upstream events of T cell activation will define whether our observation is exclusively dependent in IL-12 reduction.

Importantly we had the opportunity to verify our findings using DCs derived from a constitutive gene knockdown mouse model. VAMP7 deficient mice present pros and cons. On one hand, in the situation of chronic deletion of VAMP7, given the functional redundancy of SNARE proteins, we expect major compensatory events that may mask the functional impairment observed in the DCs with acute SNARE-depletion. On the other hand, the study of untreated cells can provide more reliable informations about the behaviour of “healthy” cells, not affected by the stressful and toxic process of siRNA transfection and with a complete protein depletion.
Notably we confirmed that VAMP7 KO DCs secrete less IL-12p70 than their wild type counterparts. This finding reinforces the siRNA data ruling out the possibility of any off-target effect or other not-specific toxic effect. The extent of reduction in VAMP7 KO DCs was similar to that in VAMP7-silenced DCs, thus indicating that compensatory mechanisms act similarly during acute depletion and constitutive knock out.

In contrast, IL-6 levels in VAMP7 KO DCs were similar to those measured in wild type mice. This can be interpreted as a compensatory phenomenon occurring in KO that abrogates the little decrease observed in VAMP7-depleted DCs. Therefore combined analysis of data from silenced and KO cells indicate that dependency on VAMP7 for IL-12 is more pronounced than for IL-6.

Importantly, CD8+ T cell priming assays confirmed a decrease in the amount of IFN-γ secreted by cells primed by VAMP7 KO DCs. This completely rules out the possibility that the reduction observed using silenced cells might have been caused by differences in survival of silenced DCs and strengthen the hypothesis that reduced IL-12p70 (and not reduced IL-12 p40 or IL-6) is indeed responsible of the effect.

Facilitated by the use of VAMP7 KO cells we are now planning to perform in vitro assay to measure synaptic release, and in vivo assays to follow long term T cell programming.

In parallel with VAMP7, we have investigated the role of VAMP3 in cytokine secretion in DCs. VAMP3 silencing was very efficient and led to a reproducible almost complete knockdown of protein. Stimulation of VAMP3 depleted cells with CpG-B showed a strong inhibition in the production of all the cytokines tested. This finding hampered subsequent analysis of a possible role of this SNARE in cytokine transport and raised the hypothesis that VAMP3 is involved in upstream events of activation. It is known that the molecular regulator triggering upregulation of inflammatory cytokine transcription in the TLR-mediated signalling is NF-kB (Janeway and Medzhitov, 2002). In pDCs it has been shown that the TLR9-dependent activation of this pathway occurs specifically in the late endosomes (LE), and that the agonist CpG B reaches these compartments rapidly passing from the plasma membrane through early endosomes (EE) via vesicular transport (Guiducci et al., 2006). Also the TLR9 itself is transported, after new synthesis, from the endoplasmic reticulum to the VAMP3+ EE via vesicular traffic, and from the EE to the LE in a trafficking dependent on the adaptor protein AP-3 (Sasai et al., 2010). We can hypothesise that in cDCs a similar trafficking mechanism is present, and that the early trafficking of CpG B or TLR9 in the EE may be affected by VAMP3 loss. Future experiments will include the analysis of different stimuli to establish whether unresponsiveness is TLR9 specific or not and biochemical analysis to identify the signalling pathways affected by VAMP3-depletion.
For an efficient T cell priming, DCs must efficiently produce all the 3 main signals, i.e. antigen-loaded MHC, co-stimulatory molecules and cytokines. Among the different DCs subsets, CD8α⁺ DCs are specialized in antigen cross-presentation and IL-12 production and most efficiently induce CTLs responses.

Having identified SNAREs as trafficking proteins required for DCs activation and cytokine secretion I focused in the second part of this thesis on investigating their potential role in a pathological context. It has been widely documented that DCs in tumor bearing host (or exposed to tumor derived factors) lose the ability to produce inflammatory cytokines and become tolerogenic. A skewed cytokine secretion profile during antigen presentation in cancer contributes largely to tumor escape. Nevertheless, the molecular basis of suppression in DCs have not been extensively studied.

To investigate the hypothesis that SNAREs may be targeted during suppression we established a model of lung cancer that was used to study cytokine suppression in DCs isolated from the primary tumor site, from the spleen or co-cultured with lung cancer cells ex-vivo. The choice is motivated by previous studies indicating that transwell co-cultures of DCs with the Lewis lung carcinoma cell line cause a decrease in IL-12 secretion and an increase in the secretion of the cytokine IL-10 (Liu et al., 2009).

We first confirmed in trans-well experiments that 72 hrs of exposure to tumor-derived soluble factors were enough to cause a strong downregulation of IL-12 production in CpG-B stimulated DCs. We could not evaluate differences in IL-10 as the levels of the cytokine were below the detection limit in this system.

As the concept of DCs cytokine suppression comes mostly from ex-vivo experiments or from studies using tumors of different origins and often implanted in ectopic sites we decided to extend the analysis to endogenous DCs in mice bearing lung tumors implanted in the their natural target organ (in the lungs as opposed to subcutaneous).

The spleen was recently described as a crucial reservoir of myeloid suppressor cells and the main organ in which T cell tolerance is established [Ugel, 2012 #561; Cortez-Retamozo, 2012 #562;]. Moreover, DCs isolated from host bearing EL-4 thymoma were shown [Herber, 2010 #150] to lose
T cell priming capacity because of reduced antigen processing, although the authors did not measure cytokines production in this context.

In the spleen of mice bearing lung tumors we observed an expansion in the Gr-1<sup>hi</sup>/CD11b<sup>hi</sup>/CD11c<sup>lo</sup> population in agreement with previous report (Ugel et al., 2012). Instead conventional DCs did not show any major difference in subset composition (CD8α<sup>+</sup> and CD8α<sup>-</sup> fraction) or maturation level. Despite the lack of any phenotypic change, IL-12 production upon CpG-B treatment was strongly affected as compared to DCs isolated from control mice. The impairment was more evident in mice bearing end-stage tumours suggesting a progressive expansion on the DC immunosuppression, raising from the lungs and extending to the peripheral lymphoid organs.

In the context of the primary tumor we made some new observations beyond our original scope. We identified myeloid cell subsets based on a gating strategy established to study DCs during influenza virus infection, that take into account the complexity of the DCs subset in the lung and the presence of an abundant population of macrophages that express the CD11c marker. We observed a dramatic increase of both CD11c<sup>+</sup> and CD11c<sup>-</sup> macrophages, likely a mixture of TAM and MDSC that needs to be further defined. Within DCs, we observed an increase in the percentage of CD11c<sup>hi</sup>/CD11b<sup>hi</sup> cells, with levels of Gr-1 remaining low and with no substantial changes in the intensity of MHC-II staining. Interestingly we saw a decrease in the CD103<sup>+</sup> DCs, migratory cells most implicated in the cross-priming of CD8<sup>+</sup> T cells (del Rio et al., 2007). This is reminiscent of what happens during influenza virus infection where CD103<sup>+</sup> cells were shown to rapidly migrate towards the draining lymph nodes for the antigen-presentation (Helft et al., 2012). Further studies on mediastinal and axillary lymphnodes composition will elucidate whether this impairment observed in tumor-bearing lungs is due to an elevated migration or simply to an increase in cell death of this particular subtype of DCs. Furthermore, in contrast with what stated in other tumor models, the total DC population present in tumor-infiltrated lungs didn’t show a regression towards a more immature phenotype, as MHCII levels observed remained unchanged even in very late stages of tumor progression.

As far as our primary objective was concerned we confirmed that IL-12 production is strongly impaired in DCs isolated from the lung of diseased animals as compared to healthy controls. In tumor-associated DCs we could also detect an increase in IL-10 levels.

Most remarkably, analysis of SNARE expression in the three models (ex-vivo co-culture, splenic DCs and tumor associated DCs), revealed an altered expression of SNARE proteins. Although the data are still preliminary and more replicates are needed to establish the behavior of all SNAREs we
can already conclude that VAMP3 is consistently reduced in all cases. VAMP3 reduction was also clear at the protein level in tumor associated DCs. This original observation coupled to our work in cells depleted in VAMP3 by gene silencing strongly suggest that this may represent a novel mechanism used by cancer cells to dampen DCs capacity to respond to innate signals. Moreover, VAMP3 may be necessary to accomplish other important DCs functions such as endocytosis and phagocytosis and antigen processing, all issues that will be addressed in future studies.
5. Conclusions
In conclusion, this work sheds light on the molecular bases of cytokine secretion in DCs, a fundamental aspect of DCs biology that has been poorly characterized so far.

We have identified the late endosomal SNARE VAMP7 as a regulator of the intracellular trafficking of IL-12 towards the immunological synapse. Our evidences pointing to a role of VAMP7 in cytokine secretion can be summarized as follow: 1) VAMP7 is upregulated during TLR9-dependent DC maturation; 2) VAMP7 and IL-12p70 are co-recruited at the IS where they are found to overlap in vesicles underneath the DCs membrane facing the T cell; 3) VAMP7 depletion by siRNA or constitutive gene knock-down resulted in a decrease of IL-12 release upon TLR9 triggering; 4) priming of IFN-γ producing CD8+ T cells upon interaction with VAMP7-depleted DCs is impaired.

Second, we show that VAMP3 is a potential regulator of the signalling of TLR9, as its depletion leads to a strong decrease in the cytokine production upon CpG B stimulation.

Third, we made the original observation that SNAREs, particularly VAMP3, are downregulated in tumour-educated DCs. This implies the existence of a yet uncovered new mechanism of tumour escape by targeting the expression of trafficking proteins in DCs.

Collectively these data are of interest for the fundamental understanding of the mechanisms regulating cytokine, and more specifically IL-12 secretion, in DCs under normal and pathological conditions.
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