The role of Wiskott-Aldrich Syndrome protein-mediated actin dynamics in controlling type-I IFN production in plasmacytoid dendritic cells

Candidate: Francesca Prete

Thesis supervisor: Dr. Federica Benvenuti

XXIV cycle – Academic Year 2011
Alla famiglia,

ad Eu, Fabio, Giulia, Michela, Roberta, Gabriele.

A chi, anche se lontano, resta.
In everything I seek to grasp

The fundamental:
The daily choice, the daily task,
The sentimental.

To plumb the essence of the past,
The first foundations,
The crux, the roots, the inmost hearts,
The explanations.

And, puzzling out the weave of fate,
Events observer,
To live, feel, love and meditate
And to discover.

Boris Pasternak
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Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by recurrent infections, and a marked predisposition to develop autoimmune phenomena. The disease is caused by mutations in WASp, a key regulator of actin polymerization expressed only in hematopoietic cells. A general impairment of hematopoietic cell functions contributes to the pathogenesis of the disease. Neutrophils, B cells, T cells and DCs deficient for WASp were all shown to have impaired homing ability, a cellular function that strictly depends on spatio-temporal regulation of actin polymerization. WASp null T cells present severe impairment in coupling TCR stimulation to proliferation and fail to organize signaling molecules within the immunological synapse (Badour et al., 2004; Dupre et al., 2002; Sims et al., 2007). B cells intrinsic defects include altered B cell receptor clustering, defective homeostasis of mature B cells and a specific reduction in the expression of the complement receptor (Park et al., 2005; Simon et al., 1992; Westerberg et al., 2001). WASp null DCs fail to assemble podosomes and display late migration from the periphery to lymph nodes (de Noronha et al., 2005). Moreover, WASp expression in DCs is critical to organize the dynamic cytoskeletal changes that facilitate DC-T cell interaction during antigen presentation (Pulecio et al., 2008; Bouma et al., 2011). Together, these cellular alterations provided clues to understand the reduced response to pathogens and the immunodeficiency of WAS patients. However, the mechanisms by which perturbation of actin dynamics promote autoimmune phenomena are less clear.

Autoimmune complications occur in 40-72% of children with severe WAS phenotype. The most common autoimmune features that develop in WAS patients include hemolytic anemia, vasculitis, renal disease, and arthritis (Dupuis-Girod et al., 2003; Sullivan et al., 1994; Humblet-Baron et al., 2007). Impairment of T and B cell tolerance have been reported in WAS patients and in Was<sup>-/-</sup> mice, but the exact cellular mechanisms that link loss of WASp function to autoimmunity have not been fully elucidated yet (Becker-Herman et al., 2011; Recher et al., 2012; Marangoni et al., 2007; Maillard et al., 2007; Humblet-Baron et al., 2007).

It is increasingly recognized that excessive activation of pDCs and elevated type-I interferon (IFN) levels are pathogenic in several human autoimmune diseases such as SLE, psoriasis, Sjogren’s syndrome. Since WAS autoimmune manifestations partially overlap with those of type-I IFN diseases, we hypothesized that pDCs/IFN-α axis may have a role in WAS-associated autoimmunity.
In the first part of results we present the analysis of the pDC compartment in a mouse model of the disease, a mouse knock-out for WASp (WKO mice). We show that pDCs from WKO animals are chronically activated, secrete type-I IFN constitutively and become refractory to further stimulation in vivo. By depleting WASp expression or by interfering with actin dynamics in pDCs we prove that WASp-mediated actin dynamics control the activation of the TLR9/IFN-α pathway in a cell autonomous fashion.

Based on the results of previous section, we speculated that WASp may regulate IFN-α production by controlling the correct organization of the endocytic pathway. It is well known, in fact, that type-I IFN production relies on spatio-temporal regulation of TLR9 signaling in the endocytic pathway, at the same time, it is largely established that many steps of the endocytosis are regulated by actin regulatory proteins of the WASp family. Therefore, we proceed presenting a series of experiments exploring the role of WASp in mediating signaling downstream TLR9 activation in pDCs. Tracking of TLR9 agonist in pDCs show that WASp controls cellular architecture and early endosomes size leading to accumulation of large aggregates of TLR9 agonist in WASp-deficient cells.

To demonstrate a link between the alteration in the pDCs/IFN-α axis found in WKO mice and the pathogenesis of WAS autoimmunity, we first analyzed the presence in patients of clinical aspects known to be associated with type-I IFN diseases. We report that WAS patients display a moderate type-I interferon signature, indicating an exposure to elevated levels of this cytokine. Finally, we moved back to the mouse model of WAS to start investigating the presence of alterations of innate/adaptive immunity classically attributed to unrestrained type-I IFN production. In line with the well known inducing effects of systemic type-I IFNs, our preliminary data illustrate a generally more activated phenotype of immunocytes in WKO mice.

In sum, our work provide 1) the first demonstration of an altered pDCs/IFN-α axis in WAS, 2) the existence of a cell intrinsic mechanism of increased pDCs activation in WASp-null pDC, and 3) a new role for actin in restraining excessive activation of TLR9 in pDCs.

These observations add a new layer of complexity to our understanding of the pathophysiology of WAS, and raise important considerations about the treatment of patients with autoimmune phenomena.
INTRODUCTION
1.1 Innate immunity vs adaptive immunity

The immune system has evolved under a strong selective pressure imposed by pathogens. As a result, all multicellular organisms have developed the ability to recognize invading microbes and to eliminate them efficiently without causing damage to self. The mammalian immune system in particular effectively fights infection through the cooperation of two well connected systems, innate and adaptive immunity, each of which uses different strategies to recognize microbial invaders. Specifically, the innate immunity detects infections using few germ-line-encoded pathogen-recognition receptors (PRRs) that recognize certain molecular structure unique to classes of infectious microbes, while the adaptive immunity uses clonally expressed and randomly generated receptors of limitless specificity (Medzhitov and Janeway, 1997; Cooper et al., 2006). PRRs are strategically expressed on cells that are the first to encounter pathogen during infection, such as surface epithelia, and also on innate effector cells known as antigen presenting cells (APCs), such as dendritic cells (DCs). The direct recognition by PRRs of specific pathogen-associated molecular patterns (PAMPs) triggers cell biological processes such as endosomal and cytoskeletal dynamics and induces the expression of innate response genes, including those encoding co-stimulatory molecules, pro-inflammatory cytokines and chemokines. This initial and rapid inflammatory response leads ultimately to the activation of adaptive immunity by recruiting and activating pathogen-specific B and T cells that are instructed to limit pathogen spread and eradicate the invaders.

In the last years, various classes of PRRs have been discovered in vertebrates, including the Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), as well as some members of the C-type lectin family (Pichlmair and Reis e Sousa, 2007). Among them, the TLR family is the most extensively studied and characterized.

1.1.1 Toll-like receptors

TLRs are a major class of PRRs in sensing pathogens. Ten and 12 TLRs have been identified in humans and mice, respectively, with TLR1-TLR9 being conserved in both species. TLRs can be divided in two subgroups depending on their cellular localization and respective PAMPs ligand. The first group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which are expressed
on cell surface and recognize mainly microbial membrane components such as lipoproteins, lipids and proteins. The second group is composed of TLR3, TLR7, TLR8 and TLR9, which are expressed intracellularly on vesicles such as endosomes, lysosomes and endolysosomes, where they recognize microbial nucleic acids (Iwasaki and Medzithov, 2004).

TLRs differ from each other in ligand specificities, expression patterns and in the target genes that they can induce. TLR4 play a critical role in the recognition of bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, whereas TLR2 is pivotal to the recognition of bacteria peptidoglycan and lipoproteins, but is also able to bind components from fungi (zymosan) and parasites (tGPI-mucin from Trypanosoma cruzi). Other TLRs are involved in the recognition of microbial proteins such as flagellin by TLR5 or apicomplexan profilins by TLR11. TLRs have also an essential role in the antiviral immune responses due to their ability to induce type I IFNs, a family of cytokines specialized to induce and to coordinate immunity to 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-cytidyllic acid (poly(I)•poly(C)) (Kawai and Akira, 2006).

Upon recognition of their specif ligands, TLRs transduce signals through two pathways involving distinct adaptor proteins containing Toll/IL-1R (TIR) domains. One of these adaptors, MyD88, is used by all of the known TLRs except TLR3. MyD88 is recruited to the receptor-ligand complex in an interaction that is mediated by its C-terminal TIR domain. Through its N-terminal the adaptor engages the kinase IRAK4, thereby allowing the association of IRAK1. IRAK4 then

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**Figure 1. MyD88-dependent TLR signalling.** All TLRs use MyD88 with the exception of TLR3. MyD88 binds the TIR domain of the receptor and phosphorylates IRAK4 which in turn phosphorylates IRAK1. IRAK1 phosphorylates TRAF6 leading to the ubiquitination of TAK complex. Activation of IKK and MAPK pathways leads inflammatory and antiviral responses.

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induces the phosphorylation of IRAK1 that associates with TRAF6, leading to the activation of two distinct signalling pathways: NF-kB and MAPK pathways (Janeway and Medzhitov, 2002). In detail, upon their phosphorylation IRAK1 and TRAF6 dissociate from the receptor and form a complex with TAK1, TAB1 and TAB2 at the plasma membrane, which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded and the remaining complex translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 and UEV1A. This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both MAPK and the IKK complex. (Figure 1).

TLR3 and TLR4 are able to activate NF-kB in a MyD88 independent way that involves the adaptor molecule TRIF and other signalling molecules such as RIP1 and TRAF6. Triggering of TLR3 and 4, through the adaptor TRIF, induces also the production of type-I IFN. TRIF associates with the kinases TBK1 and IKKi which mediate the phosphorylation and the translocation into the nucleus of IFN-regulatory factor 3 (IRF3). Also TLR7, 8 and 9 elicit type-I IFN induction, in particular IFN-α, through MyD88, in a pathway that involves the kinases IRAK1 and IRAK4 and the IFN-regulatory factor 7 (IRF-7) (Trinchieri et Sher, 2007).

Collectively, the induction of these signalling cascades induce the expression of a variety of host defense genes that include those encoding for inflammatory cytokines (IL-6, IL-12 and TNF-α) and type-I IFN cytokines, the up-regulation of co-stimulatory molecules (CD40, CD80 and CD86) and the altered expression of chemokines receptors (CCR2, CCR5 and CCR7) (Akira et al., 2001).

1.2 Dendritic cells general features

The key cellular player in translating innate information into adaptive immunity are the members of the heterogeneous dendritic cells (DCs) family. DCs express a large repertoire of PRRs and, in response to signals from these receptors, undergo a complex phenotypic and functional transformation, known as “DC maturation”. Mature DCs are immunogenic antigen-presenting cells (APCs) competent to sustain the expansion and differentiation of antigen-specific T cells into effector cells.

DCs have evolved several features that greatly enhance their capacity as APCs. Among these, the ability of immature DCs to efficiently take up particles and pathogens by phagocytosis and the
property to form large pinocytic vesicles to sample extracellular fluids and solutes in a process called macropinocytosis (Trombetta and Mellman, 2005). Moreover, DCs express a great variety of endocytic receptors that belong to several families and are involved in antigen uptake. Examples of these endocytic receptors include receptors for the Fc portion of immunoglobulin (FcRs) and complement receptors (CRs), that are involved in the internalization of particle that are opsonized by immunoglobulins or complement factors, respectively, as well as scavenger receptor (SRs) and C-type lectin family receptors (CLRs), that directly recognize specific structures on both self-antigens and pathogens (Trombetta and Mellman, 2005).

After their entry into DCs, antigens are degraded into small immunogenic epitopes that associate with the major histocompatibility complex molecules (MHC) and are transported to the DC plasma membrane where they ultimately trigger the activation of naive T lymphocytes. Specifically, the activation of CD8+ and CD4+ T lymphocytes requires recognition by the T cell receptor (TCR) of fragments of antigens (peptides) loaded on MHC class I and MHC class II molecules, respectively. The antigen processing pathways that lead to the formation of peptide-MHC complexes, rely on conserved proteolytic mechanisms (proteasome, lysosomes) that in DCs were turned towards optimal T cell stimulation (Banchereau and Steinman, 1998).

Initial studies on antigen presentation defined a strict compartmentalization of MHC class I and II biogenesis, with MHC class II molecules encountering exogenous antigen in the endocytic pathway and MHC class I loading with endogenous antigens in the endoplasmic reticulum (ER). Although still valid, recent evidences proved some exceptions to these antigen presentation pathways by showing that MHC-II molecules can present intracellular antigens not coming from extracellular space and MHC-I can present peptides derived from exogenous antigens, in a process generally referred to as cross-presentation (Heath et al., 2004).

### 1.2.1 The “Langerhans cells paradigm”

In 1970s Steinman and colleagues described DCs as following a life cycle that was modeled according to observations of studies on DCs found in the epidermis (Langerhans cells, LCs) (Villadangos and Schnorrer, 2007). This model, known as “Langerhans cell paradigm”, proposes that DCs can exist in two functional states: immature and mature. In the periphery, immature DCs efficiently internalize self and non self antigens but are inefficient in presenting them to T cells. Only upon the interaction with the antigens and PRR triggering DCs undergo maturation with resulting endocytic activity downregulation, activation of the antigen processing machinery and increase of T-cell co-stimulatory molecules. In parallel, modifications in the pattern of chemokine
receptors and adhesion molecules and changes in the cytoskeleton organization cause the mobilization from the periphery to secondary lymphoid organs where the antigen are efficiently presented to T cells thus initiating adaptive immune responses.

However, in the last few years a far greater complexity it has been found among the DCs system, with the consequent need to add modifications to this original DCs life cycle paradigm. It is, for instance, now clear that only a fraction of the DCs found in lymph nodes are derived from cells previously resident in the peripheral tissue. In a steady state condition, in absence of microbial infections, half of the DCs found in lymph nodes and most of the DCs in the spleen are resident cells, with an immature phenotype and still great efficiency in antigen uptake and processing. Therefore, these lymphoid-organ-resident DCs do not conform to the Langerhans cell paradigm, since they develop from bone marrow precursors within the lymphoid organs and without previously trafficking through peripheral tissues (Henri et al., 2001; Wilson et al., 2003).

1.2.2 DCs classification

The phenotypic and functional analysis of the DCs found in thymus, spleen and lymph nodes has, therefore, shown such a large heterogeneity among the DCs network that it was made necessary a strict categorization. Therefore, some main classification criteria have been identified: first, DCs can be classified either as precursors of DCs (pre-DCs) or the DCs products themselves (conventional DCs, cDCs); second, on the base whether the DCs migrate from the peripheral tissues via the lymphatics to the draining lymphoid organs (migratory) or develop and reside within the lymphoid organs (resident); third, whether they are already present in steady state conditions or whether they arise after infection or inflammation (inflammatory) and fourth, into the different subtypes.

1) DC stage: Pre-DCs versus conventional DCs (cDCs)

Cells that are in the immediate previous step of DC development are classified as pre-DCs. They can differentiate into DCs automatically in the steady-state or require additional signals given by infectious or inflammatory events. Examples of pre-DCs are plasmacytoid DCs (pDCs) and the precursors of steady-state splenic cDCs. By contrast, conventional DCs (cDCs) have a dendritic form and show DC functions already at steady-state (Shortman and Naik, 2007).

2) DC life history: Migratory versus resident DCs
Migratory DCs arise from the periphery, act as sentinels for microbes or peripheral self-antigens, and then migrate via the lymphatics toward draining lymphoid organs. This process goes together with DC maturation and occurs at basal rate in the steady-state conditions, so that they are found in the lymphoid organs as mature DCs. For instance, Langerhans cells (LCs) and interstitial DCs belong to the migratory DC category.

Resident DCs are localized in all lymphoid organs of the mouse, where they are found in a MHC-II\textsuperscript{int} immature state, which allows their distinction from the mature MHC-II\textsuperscript{hi} migratory DCs. Resident DCs develop from bone marrow precursors within the lymphoid organs without previously trafficking through peripheral tissues. They include the CD11\textsuperscript{c}\textsuperscript{hi}CD45RA\textsuperscript{lo}MHC-II\textsuperscript{int} cDC, which can be further broken into two subsets, the CD8\textsuperscript{+} cDC and the CD8\textsuperscript{-} cDC (Villadangos and Schnorrer, 2007) (Figure 2).

3) Host state: Inflammatory DCs

Inflammatory DCs represent a novel DC population that is not found in the steady state, but appears only as a consequence of infection or inflammation. One example is given by Tip DCs that appear in the spleen of mice infected with Listeria monocytogenes and are specialized in the production of tumor-necrosis factor (TNF) and inducible nitric-oxide synthase (iNOS). Tip DCs are CD11c\textsuperscript{int}CD11b\textsuperscript{hi}MAC-3\textsuperscript{+}, unlike splenic steady-state cDCs that are CD11c\textsuperscript{hi}CD11b\textsuperscript{low}MAC-3\textsuperscript{-}, and their development is dependent on the expression of the chemokine receptor CCR2. There are recent in vivo evidences which would prove the origin of this inflammatory DCs subset from monocytes released after infection from the BM and recruited to enter the blood and lymphoid organs via CCR2 (Taylor and Gordon, 2003).
4) DC subtypes:

Conventional DCs (cDCs) subtypes

Among conventional DCs, two main categories can be distinguished based on the path that they follow to access the lymphoid organs: migratory DCs and lymphoid-tissue resident DCs. Migratory and lymphoid-tissue-resident cDCs can be further divided into distinct subtypes. In the case of migratory cDCs, there is the classification on the base of the peripheral tissue of origin. So, the epidermal LCs are the migratory cDCs coming from skin epidermis, whereas, the interstitial DC represent a subset of cells migrating from the derma. The two subsets can be readily distinguished by the selective expression of the C-type lectin langerin on the surface of epidermal LCs and by LC-specific intracellular organelles known as Birbeck granules (Kissenpfennig et al., 2005). LCs and dermal DCs are characterized by the expression of many PRRs and endocytic receptors and are able to efficiently mediate the uptake of macromolecules and a broad range of microorganisms (Valladeau and Saeland, 2005). Residing in most peripheral tissues at site of interface with the environment, migratory DCs constitutively take up antigens and traffic to lymph nodes where they interact with both CD4^+ and CD8^+ T cells. Indeed, cutaneous DCs express upon activation high levels of MHC-I and MHC-II molecules and are able to efficiently prime both T helper and cytotoxic T lymphocytes. On the other hand, their ability to cross-present soluble or cell-associated antigens is less defined. In a mouse model of autoimmunity, where the ovalbumin (OVA) class I peptide is expressed under the control of the human keratin 14 promoter (K14-OVAp mice), LCs have been demonstrated to cross-present this highly expressed antigen in the skin draining LNs and to induce the expansion of antigen specific cytotoxic T cells endowed with effector functions (Mayerova et al., 2004).

Lymphoid-tissue resident DCs constitute half of the lymph node DCs (the second half is given by migratory DCs) and all the DCs found in the spleen and thymus (Wilson et al., 2003). They are further subdivided into three subsets depending on the expression of CD4 and CD8 markers: CD4^+CD8^+, CD4^-CD8^+ and CD4^-CD8^- cells. In contrast to migratory DCs, resident DCs mantein an immature phenotype in steady state, but can be induced to mature by inoculation of inflammatory stimuli. Thus, the function of the immature lymphoid organ DCs may be to promote tolerance in steady state while maintaining their capacity to respond to infections reaching those organs (Wilson et al., 2003). So, due to their crucial role in the control of immunity, the definition of the in vivo relevance of the various subsets of lymphoid organ resident DCs in terms of antigen presenting functions has been subject of intense study. Both in vitro and in vivo analysis of the ability of resident DCs subsets to present exogenous antigens on MHC-II or cross-present them on MHC-I
have revealed a functional dichotomy between the CD8$^+$ and CD8$^-$ (comprising both CD4$^+$ and double negative DCs) subsets of resident DCs. Specifically, it has emerged that the CD8$^+$ DCs are the most efficient at cross-presenting cellular and soluble antigens (Schnorrer et al., 2006). Furthermore, CD8$^+$ DCs have also been identified as the major subset involved in the MHC class I presentation of antigens from viruses (HSV1, influenza and vaccinia virus) and cytosolic bacterium \textit{L. monocytogenes} (Belz et al., 2005). By contrast, CD8$^-$ DCs seem to be more efficient at presenting exogenous antigens on MHC-II molecules, especially in the case of phagocytosed antigens and soluble antigens (Schnorrer et al., 2006), and in activating humoral responses (Corbett et al., 2005).

A further classification of cDCs can be made on the base of CD103 (αE) integrin expression. Distinct CD103$^+$ DCs subsets can be found in various non-lymphoid and lymphoid compartment. For example, in the spleen, between 50% and 70% of splenic CD8$^+$ DCs co-express CD103. Splenic CD8α$^+$,CD103$^+$,CD207$^+$ DCs play a dominant role in phagocytosis of blood-borne apoptotic cells and show potent activity in cross-presentation. A transient ablation of this subset causes failure of tolerance induction to cell-associated Ags so indicating a pivotal role in tolerance induction by apoptotic cell clearance (Qiu et al., 2009). In the gut, CD103 is expressed by CD11c$^{hi}$MHC-II$^{hi}$ DCs in the villus of small intestine, and by DCs present in mesenteric lymph node (MLN), lamina propria and Peyer’s patches. Conditioned by epithelial-derived factors, MLN CD103$^+$ DCs deriving from lamina propria have been shown to induce gut-homing molecules on effector T cells and the generation of FoxP3$^+$ Tregs required for the development and maintenance of tolerance to not dangerous non-self-antigens (Coombes et al., 2007). Finally, in the steady state conditions, 40-60% of DCs residing in the lung-draining lymph nodes express CD103. They migrate in a CCR7-dependent manner to the bronchial lymph nodes to cross-present harmless antigen to CD8$^+$ T cells (del Rio et al., 2007). Furthermore, CD103$^+$ DCs have been shown to mobilize in response to influenza virus infection to efficiently induce CTL activity by CD8$^+$ effector T cells (Kim and Braciale, 2009).

Both human and mouse immune systems have at least one subset of migratory cDCs in the dermis and a subset, that of Langerhans cells, in the epidermis. A notable discrepancy between the two systems has been found at the level of the resident DCs populations in lymphoid tissues. No human DCs expressing CD8 had been in fact observed, and only recently, studies started making progresses toward the identification of the human counterpart to mouse CD8$^+$ DCs (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). These studies were focused on the human blood CD141 (BDCA3)$^+$ DCs. This subset was found to express the chemokine receptor XCR1 (Bachem et al., 2010; Crozat et al., 2010), a molecule selectively expressed by murine CD8$^+$
DCs (Dorner et al., 2009), and the transcription factors Batf3 and IRF-8 (Jongbloed et al., 2010, Poulin et al., 2010), typical markers of mouse CD8\(^+\) DCs. Moreover, CD141\(^+\) DCs lack expression of IRF-4, a factor required for development of some murine DCs subtypes, but not CD8\(^+\) DCs.

Finally, CD141\(^+\) DCs were capable to phagocytose dead cells and cross-present cell-associated and soluble antigens (Jongbloed et al., 2010, Poulin et al., 2010). The special ability of CD8\(^+\) DCs in cross-presentation was, however, not clearly mirrored by CD141\(^+\) DCs. In one study, CD141\(^+\) DCs were slightly more efficient to cross-present than were monocyte-derived DCs (Poulin et al., 2010), whereas, in the others, CD141\(^+\) DCs appeared to cross-present more efficiently than pDCs or conventional CD1\(^+\) DCs (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010), even if the differences were not so marked as in the mouse system.

**Plasmacytoid dendritic cells (pDCs) subtype**

Plasmacytoid dendritic cells (pDCs) are a small subset of DCs present in both in human and mice with a unique ability to rapidly secrete massive amounts of type I interferons after viral infection. Differently from cDCs, pDCs constitutively express the IFN regulatory factor 7 (IRF-7) that allows the fast secretion of vast amounts of IFN-α in a signaling pathway initiated by TLR7 and TLR9 engagement. In recent years, pDCs have become object of intensive studies that centered them as pivotal in controlling immunity and diseases, both because their IFN-α production capacity and antigen presenting properties.

<table>
<thead>
<tr>
<th>Features</th>
<th>Lymphoid-organ-resident DC subsets</th>
<th>Migratory DC subsets</th>
<th>Monocyte derived</th>
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<td></td>
<td>CD4(^+) DCs</td>
<td>CD8(^+) DCs</td>
<td>DN DCs</td>
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<td><strong>Surface markers</strong></td>
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<td>CD11b</td>
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<td><strong>Functional features in the steady state</strong></td>
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Table 1. Mouse dendritic-cell subsets
1.3 Plasmacytoid dendritic cells (pDCs)

As previously described, pDCs are a small population of DCs specialized in the secretion of type-I interferons (IFNs). These cells were first described in humans as plasmacytoid T cells or plasmacytoid monocytes because of their plasma cell-like shape, expression of CD4, and their expression of monocytic markers such as the α chain of the interleukin-3 receptor (IL-3R) and CD68 (Facchetti et al., 1988). Initially the function of these cells was unknown, however, the presence of a large ER suggested a potential pivotal role in cytokine secretion. Later, Liu et al. showed that plasmacytoid T cells/plasmacytoid monocytes were able to induce T helper type 2 (Th2) cell differentiation in response to stimulation with CD40 ligand, so that the name of type 2 DC precursor (pre-DC2) was proposed (Farkas et al., 2001). Eventually, pDCs were found to secrete type I IFN and to correspond to the human natural IFN-producing cells (NIPCs), a small subset of blood cells that had been previously shown to produce massive amounts of type I IFN after stimulation in vitro with several viruses (Siegal et al., 1999). The mouse cells with phenotypic and functional features comparable to those of human pDCs were identified in 2001 (Nakano et al., 2001).

1.3.1 PDCs in the immune response: general features

PDCs provide an initial line of host defense against viral infection. PDC-derived type-I IFN enhances the ability of cDCs to mature and stimulate T cells, thus promoting antiviral CTL responses (Yoneyama et al., 2005; Le bon et al., 2003). In addition, pDCs-derived cytokines are critical for the initiation of early antiviral NK cell responses (Trinchieri et al., 1978; Bandyopadhyay et al., 1986). Furthermore, pDCs provide a critical co-stimulatory signal for the CpG-induced activation of cDCs (Shen et al., 2006; Kuwajima et al., 2006). Aside from their cytokine- and CD40L-mediated activating function to control NK and cDC activities, pDCs have been also found to play a direct role as APC in the onset of T cell stimulation. However, the potential of pDCs to prime naïve T cells remains controversial. Freshly isolated pDCs are generally poor T cell stimulators. Human blood-derived pDCs do not stimulate naïve CD4\(^+\) T cells in a MLR assay, unless cultured in the presence of IL-3 or virus (HSV)(Kadowaki et al., 2002). Moreover, murine splenic pDCs fail to induce naïve T cell proliferation to endogenous antigens, even after virus exposure (Krug et al., 2003). In contrast, murine peptide-stimulated pDCs derived from FLT-3-L bone marrow culture or spleen can promote the in vitro expansion of CD4\(^+\) T cells and Th1 cell polarization (Boonstra et al., 2003; Cella et al., 2000). It is probable that these conflicting results
depend on the different source of the pDCs used in these reports. Recently, a pDC-specific antigen-targeting strategy based on the coupling of antigens to the mAb PDCA-1 revealed that pDCs are able to trigger productive CD4$^+$ T cell responses in lymph nodes, but not in spleen (Sapoznikov et al., 2007). PDCs can also prime and cross-prime CD8$^+$ T cell responses. In mice, pDCs prime CD8$^+$ T cell when infected by cytomegalovirus (Dalod et al., 2003). In vivo, murine splenic pDCs loaded with an antigenic peptide and transferred to syngeneic recipients induce a CTL response after stimulation by influenza A virus (IAV) (Schlecht et al., 2004). In addition, pDCs were found also capable to induce efficient CD8$^+$ T cell responses against endogenous antigens, after either TLR triggering or viral stimulation (Schlecht et al., 2004; Salio et al., 2004).

Intriguingly, there is increasing evidence supporting the opposite tolerogenic role of pDCs.

In 2004, de Heer et al. found that pDCs mediate tolerance to harmless inhaled antigen by inducing T regulatory cells (Treg) that suppress the generation of effector T cells by DCs. In this study pDCs were depleted with anti-Gr-1 Ab or anti-BST-2 Ab and the asthmatic responses to OVA analyzed. The authors found that pDCs-depleted mice developed the classic features of asthma while non-depleted mice were tolerized (de Heer et al., 2004). In another study the systemic depletion of pDCs with antibodies prevented oral tolerance induced by antigen feeding. The suppression of T-cell responses was selectively mediated by liver pDCs indicating that the tolerogenic function of pDCs may be dependent on their anatomical location (Goubier et al., 2008). PDCs can induce tolerance through multiple mechanisms. PDCs express the enzyme indoleamine-2,3-dioxygenase (IDO), which promotes the catabolism of tryptophan, depleting the tryptophan source needed by T cells to generate effective responses. Expression of IDO was found to be induced after stimulation through B7 or C200R with CTLA4-Ig (Mellor et al., 2003) or CD200-Ig (Fallarino et al., 2004), respectively. Human pDCs incubated with HIV in vitro express IDO and promote the differentiation of naïve CD4$^+$ T cells toward Treg cells that suppress CD4$^+$ T cell proliferation (Boasso et al., 2007) and prevent DCs maturation (Manches et al., 2008). In addition, expression of inducible costimulator ligand (ICOS-L) on activated human pDCs can promote generation of IL-10-producing Tregs from naïve T cells (Ito et al., 2007). Finally, human pDCs can modulate T and NKT cell responses via interaction between OX40L and OX40. After culture with IL-3, human pDCs upregulate OX40L and induce Th2 polarization. After stimulation with Sendai Virus (SeV), pDCs produce type-I IFN, which overcomes the OX40L Th2 polarizing effect, resulting in Th1 rather than Th2 (Ito et al., 2004). Recently, Takagi et colleagues have established a diphtheria toxin receptor (DTR)-based mouse targeting the sialic acid binding Ig-like lectin (Siglec)-H, a pDCs-specific functional molecule, that permitted to analyze the impact in vivo of specific ablation of pDCs on tolerance (Takagi et al., 2011). To assess the role of pDCs on the antigen-specific
differentiation of CD4⁺ Foxp3⁻ T cells into CD4⁺ Foxp3⁺ inducible regulatory T (iTreg) cells, the authors adoptively transferred OT-II CD4⁺ FoxP3EGFP⁻ T cells and analyzed the generation of OT-II CD4⁺ FoxP3EGFP⁺ iTreg in wt and pDC-less mice immunized with OVA protein or CFA (complete Freund’s adjuvant) plus OVA protein. Results showed that the generation of OT-II CD4⁺ FoxP3EGFP⁺ iTreg cells was severely diminished in spleen of pDCs-ablated mice, indicating a pivotal role in vivo of pDCs in inducing peripheral tolerance under both, steady state and inflammatory conditions. In addition, results from pDC-ablated mice suggested that pDCs in the lamina propria (LP) of the small intestine control the differentiation of CD4⁺ T eff cells toward the Th1 and Th17 subsets through preferential generation and expansion of CD4⁺ Foxp3⁺ naturally occurring regulatory T (nTreg) cells, revealing a pDC-mediated regulatory loop for maintaining immune equilibrium in mucosal tissues. In vivo, pDCs were proposed to promote Treg differentiation also in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. In a recent study, Irla et colleagues used mice exhibiting a selective abrogation of MHC-II expression by pDCs to study the MHC-II-dependent antigen presentation by pDCs during EAE (Irla et al., 2010). The authors found that EAE induced by immunization with myelin oligodendrocyte glycoprotein (MOG) was severely exacerbated in mice lacking MHC-II expression by pDCs. EAE induction led to the recruitment of pDCs to lymph nodes, where they engaged in MHC-II-dependent and MOG-specific interactions with CD4⁺ T cells. This supported selective expansion of natural Treg cells able to suppress priming and expansion of pathogenic IL-17- and IFN-γ-secreting effector CD4⁺ T cells. In another recent study, Loschko et colleagues investigated antigen-presenting functions of pDCs in vivo using an antibody-mediated antigen delivery strategy (Loschko et al., 2011). Antigen delivery to pDCs via Siglec-H led to continuous low-level antigen presentation on MHC-II. This was sufficient for priming and initial proliferation of naïve antigen specific CD4⁺ T cells but it was unable to sustain CD4⁺ T cell expansion in a context of high dose of antigen and strong immune stimulation. This antigen-targeting strategy inhibited the generation of antigen specific Th1/Th17 cells without conversion to Foxp3⁺ regulatory cells. Delivery via Siglec-H of a T cell epitope derived from the autoantigen myelin oligodendrocyte glycoprotein (pMOG) efficiently delayed onset and reduced disease severity in MOG-induced EAE. In this experimental system Th cell responses were attenuated and autoantibody production inhibited. Importantly, targeting of pMOG to pDCs did not lead to a significant higher percentage of FoxP3⁺ Tregs in transferred MOG-specific 2D2 T cells in spleen and draining lymph nodes during the priming phase or in the CNS during the effector phase of EAE. Therefore, these results showed that antigen presentation by pDCs after Siglec-H-mediated delivery inhibits EAE by preventing Th cell-dependent autoimmunity in a Treg-independent manner.
Finally, a very recent study unveiled a role of pDCs in modulating immune responses against tumors showing them as pivotal players in Imiquimod(Imi)-mediated tumor cell killing (Drobits et al. 2012). Drobits et colleagues reported that after Imi stimulation dermal mast cells secreted CCL2 in a TLR7/MyD88- and IFNAR1-dependent manner, resulting in recruitment of pDCs to the treated sites. Imi-activated pDCs produced high amounts of type-I IFNs, which acted in an autocrine manner on pDCs to upregulate cytolytic molecules like granzyme B (Gzmb) and TRAIL via IFNAR1 signaling, thereby transforming pDCs into a subset of killer DCs able to directly eliminate tumor cells.

All together these findings show pDCs as a key subset in regulating adaptive immunity in vivo. As a result, intensive investigations are now focused on this cell population and on their potential role and effect in human diseases.

1.3.2 Phenotypic characterization of pDCs

The phenotypic characterization of pDCs is rather extensive. Human pDCs are distinguished from other blood cells based on the selective expression of blood dendritic cell antigen-2 (BDCA-2) and ILT7 (Rissoan et al., 2002). Human pDCs also express CD4, MHC-II, CD123 and the adhesion molecule CD2. On the base of expression of CD2 it is possible distinguish two different human pDCs subsets with distinct phenotype and function. One of these, CD2\textsuperscript{hi}, expresses lysozyme, secrete higher levels of IL-12p40 and is potent in initiating T cell immune responses (34). In addition, human pDCs express two intracellular proteins, granzyme B (Rissoan et al., 2002) and the CD2-associated protein CD2AP (Marafioti et al., 2008). On the other hands, human pDCs lack the lineage marker CD3, CD19, CD14, CD16 and the DC marker CD11c.

In mice, pDCs selectively express Siglec-H, although there is evidence that also certain macrophage subsets in the spleen, lymph nodes and brain are also Siglec-H\textsuperscript{+} (Zhang et al., 2006), and the bone marrow stromal antigen-2 (BST-2), also known as CD137 or PDCA-1 (Blasius et al., 2006). Additional pDCs markers in mice include B220, Ly6C, CD11c (at low amounts), CD8\textalpha\ (at variable amounts), whereas CD11b is either not expressed or present in very low amounts. Mouse pDCs also express Ly49Q (Omatsu et al., 2005) and the gut-homing chemokine receptor CCR9 (Hadeiba et al., 2008).

1.3.3 PDCs development
The developmental pathway of pDCs is still not fully understood. Today, Fms-Like kinase 3 ligand (FLT3L) is the only known cytokine that is critical for pDCs development from hematopoietic stem cells (HSCs) in human and mice (Blom et al., 2000; Gilliet et al., 2002). A lymphoid origin had been supported by findings that the transcripts of pre-T cell receptor α, λ5, Spi-B, as well as IgH D-J rearrangements could be found in pDCs but not in myeloid DCs (mDCs) (Rissoan et al., 2002). However, other studies have showed that FLT3+ cells belonging to either common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) could differentiate into both mDCs and pDCs in vitro and in vivo (Karsunsky et al., 2003). Additionally, studies in mice deficient for IFN regulatory factor (IRF)-8, a transcriptional factor critical for the myeloid cell lineage, revealed that the generation of pDCs, CD8α+ DCs, epidermal DCs and dermal DCs were all impaired (Aliberti et al., 2003). Recently, the concept that pDCs arise from a specific lymphoid lineage has been definitely challenged by the identification of a common progenitor able to generate classical DCs and pDCs but not monocytes in vitro (Naik et al., 2006) or in vivo (Liu et al., 2009; Onai et al., 2007). This common-DC progenitor (CDP) (Lin− CD115+ Flt3+ CD117lo) is downstream of CMP and macrophage-DC progenitors (MDP) (Lin−CX3CR1+ CD11b− CD115+ cKit+ CD135+) as adoptive transfer of either of the latter gave rise to CDP and monocytes (Liu et al., 2009). In steady state, CDPs reside in the bone marrow and locally give rise to pDCs and pre-DCs that further differentiate to cDCs and some non lymphoid tissue DCs (Liu et al., 2009; Varol et al., 2009). Finally, the identification of E2-2 as an essential and specific transcriptional regulator for pDC development has provided the evidence that pDCs start along the common DC developmental pathway, but then get diverted into a lymphocytic-like plasmacytoid state on the base of direct E2-2-mediated regulation of lineage-specific gene expression programs. The identification of E2-2 also reinforced the concept that pDCs and cDC are strictly related, as pDCs spontaneously convert into cDC-like cells in E2-2-deficient mice (Ghosh et al., 2010).

1.3.4 PDCs localization and migration

In contrast to cDCs, pDCs develop fully in the bone marrow and then enter the blood (Shortman and Naik, 2007). In the steady state, pDCs are present in the thymus and all secondary lymphoid organs (Asselin-Paturel et al., 2003; Bendriss-Vermare et al., 2001; Okada et al., 2003). Because pDCs are difficult to detect in most peripheral tissues, it is assumed that their primary route of entry into the LNs is through the blood via the high endothelial venules (HEV) and not by the lymph. However, new evidences suggest otherwise. In 2008 Pascale et al. described, in fact, pDCs
in afferent lymph of noninflamed skin of pigs and sheep in a similar proportion relative to cDCs and to that observed in the blood or the lymphoid organs (Pascale et al., 2008).

Pathogen-associated molecules or inflammatory mediators exert a dramatic effect on pDC migration, causing pDCs accumulation in the tissues from which the signals come and in the corresponding draining LNs. For instance, pDCs accumulate in the lung and mediastinal LNs of mice infected with respiratory syncytial virus (Wang et al., 2006), or influenza virus (GeurtsvanKessel et al., 2008) in the subcutaneous LNs of mice infected in the skin with Leishmania major (Baldwin et al., 2004) or in the footpad with herpes simplex virus 1 (Smith et al., 2003), and in the vaginal mucosa of mice infected with herpes simplex virus 2 (Lund et al., 2006). PDCs are also enriched in inflamed human LNs and skin lesions (Cella et al., 1999; Wollenberg et al., 2002) and are massively recruited to human and mouse skin treated with the TLR7 agonist imiquimod (Palamara et al., 2004; Drobits et al., 2012). The simultaneous accumulation of pDCs in infected tissues and draining LNs is more consistent with a role in antigen presentation or immunomodulation at sites of inflammation rather than with a role in antigen transport to the LNs for presentation to T cells. In fact, most of the pDCs that accumulate in inflamed LNs enter via HEV (Cella et al., 1999), in addition, of the pDCs recruited to peripheral infected tissues only few migrate to LNs and only relatively late after the onset of infection (GeurtsvanKessel et al., 2008).

PDCs migration property is due to the expression of L-selectin (CD62L) that interacts with L-selectin ligands expressed by HEV and stromal cells within the T cell-rich areas of lymph nodes (Penna et al., 2001). PDCs also express the chemokine receptor CXCR3, which promotes migration in response to IFN-γ-inducible chemokines such as CXCL9 and CXCL10, CXCR3 and is required for pDCs migration toward inflamed lymph nodes (Yoneyama et al., 2004). PDCs express at least three additional receptors, CCR1, CCR2 and CCR5, for the inflammatory chemokines CCL2, CCL3, CCL4 and CCL5. They also express two receptors, CXCR4 and (after activation) CCR7, for the constitutive chemochines CCL12 and CCL21. CCR7 mediate migration of murine pDCs in vitro chemotaxis assays, and CXCR4 is involved in pDCs migration in vivo in tumors (Zou et al., 2001). In addition, pDCs express the G protein-coupled receptor ChemR23, which drives their migration in response to chemerin, a peptide chemoattractant released by inflamed tissues and tumors (Vermi et al., 2005; Sozzani et al., 2010). In vitro studies have shown that CCR2, CCR5, CCR7 and CXCR3 are not functional in human pDCs (Penna et al., 2001). However, CXCR3 and CXCR4 can mediate pDCs migration when simultaneously engaged (Krug et al., 2002; Vanbervliet et al., 2003), suggesting that chemokine receptor function may require cooperation between receptors.
1.3.5 PDCs are professional type-I IFN-producing cells

PDCs are the major source of IFN-α during a viral infection. The abundant and fast type-I IFN production by pDCs is accounted for by the selective expression of specific PRRs and downstream effectors and by a peculiar compartimentalization of the signaling cascade. The binding of nucleic acid to TLR9 and TLR7, expressed intracellularly in pDCs endosomes, recruits the adaptor protein MyD88 leading ultimately to phosphorylation of IRF-7 and transcription of type- I IFN genes. In addition, the TLR7- and TLR9-triggering has been shown to be able to induce the maturation of pDCs, which consists of increased expression of MHC-II and co-stimulatory molecules (CD80, CD86 and CD40) and transcription of pro-inflammatory cytokines such as IL-6 and TNF. In 2005, Honda demonstrated the importance of a spatio-temporal regulation of the compartmentalization and trafficking through the endocytic pathway downstream of TLR9 engagement. His group showed that a prolonged retention of multimeric nucleic acid complexes, such as A-type CpG, in early endosomes provided a platform for extended activation of a molecular complex critical for the robust transcription of the type-I IFN genes, whereas the rapid translocation of monomeric nucleic acid structures to late endosomes/lysosomes failed to induce type-I IFNs production and was associated to NF-kB activation and consequent pDCs maturation (Honda et al., 2005 a).

Although pDCs activation and type-I IFN secretion are critical for an efficient antiviral response, recent studies indicate that chronic pDC activation and secretion of type-I IFN in the absence of infection may result in the establishment of a chronic inflammatory environment that predisposes to autoimmune manifestations. As a result, intensive investigations are now focused on this DCs subset and on their potential role and effects on human diseases.
Within 24 hours of activation by a virus, human pDC produce a large amount of type-I IFN (1-2 U/cell or 3-10 pg/cell), which is 100 to 1000 times more than that produced by any other blood cell type. The type IFN responses from pDC have the following features (Kadowaki et al., 2000):

1. pDC do not contain pre-existing mRNA transcripts for type I IFNs before viral stimulation.
2. Type-I IFN mRNA can be detected in pDCs as early as 4h following viral stimulation, the level reaches th peak at 12h.
3. From 6 to 12 h following viral stimulation, about 60% of total mRNA expressed by pDCs encodes for type IFNs, including all types of IFN-α, IFN-β, IFN-ω, IFN-λ and IFN-τ (Ito et al., 2006)
4. pDCs produce most IFN-α protein within the first 24h following viral stimulation
5. After the first 24 h of viral stimulation, pDCs make only moderate amounts of IFN-α, and these activated pDCs become refractory to secondary viral stimulations.

IFNα/β are produced by pDCs in response to a wide range of enveloped virus, including herpes simplex virus (HSV), Sendai virus, human immunodeficiency virus type 1 (HIV-1), influenza virus, Newcastle disease virus, and vesicular stomatitis virus, as well as parasites (*Plasmodium falciparum* (Pichyangkul et al., 2004), bacteria (e.g. *Staphylococcus aureus*) (Svensson et al., 1996), and DNA containing unmethylated CpG sequences, typical of microbial DNA (Siegal et al., 1999). Importantly, pDCs are not activated to secrete IFNα/β by all viruses and respond differently depending on the virus type. For example, pDCs are the main IFNα/β, IL-12, and TNF-α producers in response to murine cytomegalovirus (MCMV) but not lymphocytic choriomeningitis virus (Dalod et al., 2003; Dalod et al., 2002). IFNα/β induce MxA, an IFN-α-inducible intracellular protein that has been well established as a marker for local IFN-α production (Vermi et al., 2003), oligoadenylate synthetase (OAS), and double-stranded RNA (dsRNA)-dependent protein kinase (PKR), which induce cellular resistance, inhibit viral replication and block viral dissemination. Type-I IFN was found also to have an autocrine role on pDCs, consequently amplifying type I IFN secretion and inducing a refractory state to further viral infections (Kumagai et al., 2009).

In addition to type I IFN, PDCs secrete additional cytokines and chemokines following activation with pathogens or CpG oligodeoxynucleotides (ODNs) e.g., TNF-α, IL-1, and IL-6. Murine, but not human, pDCs have the capacity to synthesize bioactive IL-12 (Schnurr et al., 2004). Human pDCs, moreover, fail to produce IL-1α, IL-1β, IL-3, IL-10, IL-12, IL-15, IL-18, IFN-γ, lymphotoxin-α, or
GM-CSF at protein levels following viral stimulation. Depending on the nature of stimulus, pDCs can also secrete CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL8 (IL-8), and CXCL10 (IP-10), that are critical in recruitment of CXCR3 bearing Th1 polarized cells and NK cells, so facilitating leukocyte homing into their local action environment (Megjugorac et al., 2004).

1.3.6 Regulation of IFN synthesis in pDCs

Based on the amino acid sequence homology, IFNs have been classified into type-I and type-II. These molecules are members of the class II family of α-helical cytokines, which also includes the recently identified IFN-λ and its IL-10 homologs IL-19, IL-22, IL-20, IL-24, and IL-26 (Kotenko and Langer, 2004). Type-I IFNs include multiple members (α, β, ε, δ, κ, τ, ω), of these, IFN-α and IFN-β are the most immunologically relevant. Of note, the human and mouse genomes contain a single IFN-β gene and more than twenty diverse IFN-α genes, of which thirteen are functional. Although type-I IFNs can be produced by almost all types of nucleated cells, pDCs express a broader profile of IFNA genes than any other cell type, and, as previously mentioned, respond to a wider range of viral and non viral stimuli in synthesizing IFN-α, and more rapidly. Interferon regulatory factors (IRFs) play an important role in the regulation of IFN-α/β gene transcription. Nine cellular IRFs have been identified, and three of these IRFs are direct transducers of virus-mediated signaling. It was shown that expression of IRF-3 is sufficient to support induction of IFN-β, while IRF-5 or IRF-7 is required for activation of IFN-α gene expression in infected cells (Barnes et al., 2001). PDCs constitutively express most of the IRF genes, with an extremely high constitutive expression of IRF-7 mRNA and protein that is thought to contribute to rapid and massive IFN-α synthesis (Izaguirre et al., 2003). Splenic pDC from mice deficient for IRF-7 were shown to exhibit a marked defect in type-I IFN gene induction following infection with virus like HSV or the ssRNA Vescicular stomatitis virus (VSV) (Honda et al., 2005 b), or exposure to synthetic CpG-A ligands (Verthelyi et al., 2001). By contrast, activation was normal in pDCs deficient in IRF-1, IRF-3 or IRF-5, therefore enforcing the link between IRF-7 expression by pDCs and robust type-I gene induction.

1.3.7 TLR-mediated activation of pDCs

PDCs sense viruses through TLR9 and TLR7. TLR9 senses double-stranded (ds) DNA viral genomes rich in unmethylated CpG sequences as well as synthetic CpG ODNs. TLR7 detects viral
single-stranded (ss) RNA and synthetic oligoribonucleotides (ORNs). TLR9 and TLR7 are synthesized in the endoplasmic reticulum and transported into endosomes where TLR9 is converted into an active form by proteolytic cleavage (Park et al., 2008). In particular, TLR9 is found to be critical for pDCs to respond to DNA viruses, such as herpes simplex virus 1 (HSV-1), HSV-2 and MCMV. HSVs and Coxsackievirus B (CVB), are internalized through receptor-mediated endocytosis and delivered to TLR9/7+ endosomes (Wang et al., 2007). Other viruses, like vesicular stomatitis virus (VSV) and Sendai virus (SeV), which enter pDCs by fusion, generate replicative intermediates that are redirected from the cytosol into TLR9/7+ endosomes by autophagy. During this process, a part of the cytosol is encircled by a membrane to form a vacuole that can fuse to TLR9/7+ endosomes (Lee et al., 2007).

Unlike cDCs, human pDCs do not express TLR2, TLR4, TLR5 or TLR3, which accounts for why they do not respond to peptidoglycans, lipopolysaccharide, flagellin, or double-stranded RNA (Iwasaki and Medzithov, 2004).

Upon engagement with nucleic acid ligands, TLR9 and TLR7 deliver intracellular signals through MyD88, which acts as a docking site for a multiprotein complex containing IRAK4, TRAF6, Bruton’s tyrosine kinase (BTK) and IRF-7. More in the detail, IRF-7 is activated through ubiquitylation by the ubiquitin E3 ligase activity of TRAF6 and phosphorylation by IRAK4. Activated IRF-7 interacts with TRAF3, IKKα, IRAK1, osteopontin and phosphatidylinositide 3-kinase (PI3K), and then translocates to the nucleus where it directs the initiation of type I IFN gene transcription (Honda et al., 2005 b; Kawai et al., 2004). In parallel, TRAF6 ubiquitylates TAK1, a signal transducer that activates NF-kB and MAPKs for the induction of transcription of other pro-inflammatory cytokines, chemokines and co-stimulatory molecules (Osawa et al., 2006). As previously reported, it has been found that pDCs, differently from other cells, constitutively express high levels of most of IRFs, particularly IRF-7. The recent finding that pDCs also express low levels of the translational repressors 4E-BPs, that are crucial for regulating translation of IRF-7, might explain the pDC-specific constitutive expression of this interferon regulatory factor (Colina et al., 2008). Aside from IRF-7, IRF-5 directly interacts with MyD88 and mediates the TLR-dependent induction of pro-inflammatory cytokines such as IL-6 and TNF (Yanai et al., 2007; Yasuda et al., 2007; Takaoka et al., 2005). (Figure 4).
Two classes of CpG-containing DNA sequences induce distinct responses in pDCs

Although the importance of IRF-7 in MyD88-dependent type-I IFN gene induction is well established, it is still not fully clear why pDCs, but not other cell types such as cDCs, produce large amounts of type-I IFN. As previously mentioned, IRF-7 is constitutively expressed in greater amounts in pDCs than in cDCs. However this does not fully explain the robust type I IFN gene induction, since cDCs from mice deficient for IRF-2, that up-regulate the expression of IRF-7, remained still poor at inducing type-I genes upon triggering of TLR9 (Honda et al., 2005). In the last years, several studies started highlighting the spatiotemporal regulation of TLR7 and TLR9 signalling as one mechanism critical for the specific ability of pDCs to produce type-I IFN. These studies have demonstrated the existence of a strict spatio-temporal control of the compartmentalization and trafficking through the endocytic pathway after ligation of TLR9, with the finding of a critical link between the intracellular compartment in which the ligand-receptor interaction occurs, the physical size of TLR9 ligands and their stimulatory capacity.

Particularly, pDCs have been shown to mount a distinctive response to A-type versus B-type CpG ODNs, two classes of synthetic ODNs with hypomethylated CpG motifs. Two important features distinguish these CpG ODN, and these account for their differing ability to trigger TLR9 activation in pDCs. First, CpG-A is characterized by a polyG tail that mediates the spontaneous formation of large multimeric aggregates with a diameter of about 50nm, by contrast CpG-B is monomeric and do not form multimeric structures. Second, monomeric CpG-B rapidly traffic through early endosomes into late endosomes or lysosomes, whereas multimeric CpG-A are retained for longer periods of time in the early endosomes of pDCs (Rissoan et al., 2002; Kerkmann et al., 2005; Guiducci et al., 2006). Honda et al. have shown that the prolonged retention of multimeric CpG-A provides a platform for extensive activation of the signalling complex including MyD88 and IRF-7.
which leads to a robust and rapid type I IFN production. When CpG-A is then degraded into monomeric structures, they fail to be retained in the early endosomes and fast traffic to late endosomes. This activates a different set of signal mediators, particularly NF-kB, MAPKs and IRF-5, and leads to a distinct outcome of pDCs activation consisting in production of inflammatory cytokines, such as IL-6 and TNF, up-regulation of CD40, CD86 and MHC-II molecules, without high levels of type-I IFN production (Honda et al., 2005 a) (Figure 5). By contrast, in cDCs and macrophages, which cannot mount robust type-I IFN gene induction, CpG-A does not colocalize with transfected MyD88 or IRF-7 but localize in late endosomes/lysosomes highlighting rules governing different physical form and endosomal localization depending on the cell type.

Remarkably, elegant experiments demonstrated that manipulating the transport of CpG-A to the early endosomes of conventional DCs and macrophages induces secretion of IFN-α in similar amounts to that produced by pDCs through activation of the TLR9-MyD88-IRF-7 signalling pathway. Moreover, liposome encapsidation of CpG-B in pDCs allowed the retention in early endosomes with a subsequent massive induction of type-I IFNs, low levels of IL-6 and TNF and impaired maturation. Although the mechanisms that control endosomal trafficking in CpG- or virus-stimulated pDCs are still in great measure unknown, it is likely that a molecule(s) capable to support the endosomal longevity of TLR ligands is expressed selectively in pDCs. Further studies are, therefore, required to better characterize the critical players in directing subcellular localization of ligand-bound TLRs and adaptors, and their exact role in modulating intensity and longevity of endosomal TLR signaling.

Figure 5. Signalling of CpG ODN classes in different endosomal compartments. In pDCs, the prolonged TLR9 signalling by CpG-A aggragates from early endosomes activates MyD88 and IRF-7, wich promote strong type I IFN production. Conversely, monomeric CpG-B rapidly traffic through monomeric endosomes into late endosomes or lysosomes where induce maturation and production of IL-6 and TNF.

From Gilliet et al. 2008 Nature Reviews Immunology
1.4 PDCs-derived type-I IFNs in linking innate and adaptive immunity

Type-I IFNs derived from pDCs not only directly inhibit viral infection, but also activate the antiviral functions of cDCs, NK cells, B and T cells, and therefore initiate and orchestrate innate and adaptive antiviral immunity.

In response to HIV infection, human pDCs induce type-I IFN-dependent maturation of cDCs into potent APCs (Fonteneau et al., 2004), as well as during HSV infection pDC-derived type-I IFNs induce maturation of bystander cDCs to potently stimulate antiviral T-cell-mediated immunity (Yoneyama et al., 2005). Type-I IFNs produced by pDCs have been shown also to trigger pro-inflammatory cytokine secretion by cDCs and to induce monocytes to differentiate into DCs (Santini et al., 2000; Paquette et al., 1998). They also increase the ability of cDCs to cross-present exogenous antigens to CD8⁺ T cells and promote their clonal expansion (Kolumam et al., 2005) and their ability to induce the differentiation of naive T cells into T helper 1 (Th1) cells (Hibbert et al., 2003). In addition, Type-I IFNs derived from pDCs stimulate NK cells (Gerosa et al., 2005) and, in conjunction with IL-6, drive B cells to differentiate into mature antibody-secreting plasma cells (Jego et al., 2003).

All together these findings point to a crucial role for innate pDCs activation in the initiation of a cascade of events necessary to fight viral infections. However, if not properly regulated, pDCs activation is potentially involved in the establishment of chronic inflammation and autoimmune manifestations. In fact, in these last years, it has been largely documented that besides their beneficial antiviral properties, type-I IFNs have detrimental effects in the development, as well as in the course, of several human autoimmune diseases, like systemic lupus erythematosus (SLE), Sjorgen syndrome and psoriasis, so making pDCs attractive potential targets for future therapeutic intervention.

1.4.1 pDCs regulate the function of cDCs by type-I IFN

IFN-α/β exert multiple effects on both the innate and adaptive immune systems. A major effect is DCs activation with upregulation of MHC, especially class I MHC, chemokines and chemokines receptors, and co-stimulatory molecules, which in turn leads to efficient homing in secondary lymphoid organs and enhanced T cell responses. The ability of pDCs to induce a productive, adaptive, T cell-mediated immune response through activated cDCs were suggested in several studies conducted in the early 2000’s. Crucial are the studies conducted in systemic lupus...
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eythematous (SLE) patients, where pDCs are constantly activated through TLR9 by endogenous ligands (eg. apoptotic/necrotic material and nucleoproteins, alone or complexed with autoantibodies). In 2001 Blanco et al. demonstrated for the first time that type-I IFNs present in the sera of SLE patients strongly activates monocytes and immature cDCs, which subsequently induce strong Th1-mediated immune responses (Blanco et al, 2001). Important evidences of the type-I IFN’s activating effect on cDCs come also from HIV studies. Fonteneau et al. showed that production of type-I IFN and TNF-α by human pDCs in response to HIV infection led cDCs to upregulate co-stimulatory molecules and acquire the capacity to migrate to secondary lymphoid organs by expression of CCR7 (Fonteneau et al., 2004). This is further supported by showing that cDC lacking IFN-α receptor failed to undergo maturation during immune responses to double-stranded RNA or viral infection in mice (Dalod et al., 2003; Honda et al., 2003), and that the exposure to type-I IFN at immature stages of cDCs led to their activation and enhanced production of IL-12, IL-15, IL-18, and IL-23 (Ito et al., 2001; Luft et al., 1998; Luft et al., 1998; Paquette et al., 1998; Santodonato et al., 2003; Nagai et al., 2003; Santini et al., 2000).

1.4.2  Regulation of B cell function by pDCs

Type I-IFNs also exert a variety of effects on the development and function of B cells. Jego et al. (Jego et al., 2003) showed that, when total peripheral blood mononuclear cells from a donor vaccinated with influenza vaccine are cultured with influenza virus, large amounts of virus-specific polyclonal IgG antibodies were produced. This in vitro virus-specific polyclonal IgG antibody response could be completely eradicated by depleting pDCs from total blood mononuclear cells. This study further showed that IFN-α and IL-6 secreted by virus-activated pDCs act sequentially to drive virus-specific B cells to differentiate into plasma blasts and then mature plasma cells. Among the indirect effects exerted by IFN-α on B cells, one of the most relevant to autoimmunity is the upregulated expression of the B-lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL) by IFN-activated DCs and monocytes. BLyS and APRIL, together with T cell help and the cytokines IL-10 and TGF-β, have been shown to induce B cell differentiation to plasma cells and promote autoantibody production (Litinskiy et al., 2002). Mice transgenic for BLyS have an increased number of mature B cells and develop humoral, cellular, and histologic manifestations that resemble those seen in lupus and Sjogren’s syndrome (Khare et al., 2000; Mackay et al., 1999). Type I-IFN has been shown to exert also several direct effects on B cells relevant to the pathogenesis of systemic autoimmunity. IFN-α/β improve BCR-dependent mature B2 cell responses and increase survival and protection to apoptosis mediated by Fas (Braun et al., 2002).

Moreover, IFNAR signaling is also required for normal development and proliferation of B1 cells,
the major producers of autoantibodies (Santiago-Raber et al., 2003), and plays an inhibitory role in B cell lymphopoiesis by opposing the IL-7 response (Lin et al., 1998).

1.4.3 Regulation of T and NK cell function by pDCs

IFN-α/β are also known to exert potent effects on T cells. IFN-α/β keep activated CD8⁺ and CD4⁺ T cells alive and improve their proliferation rate by acting either directly (Marrack et al., 1999) or indirectly through induction of IL-15 by APCs (Zhang et al., 1998). Type-I IFN-induced DC maturation is also critical in promoting cross-priming of CD8⁺ T cells against virally infected targets (Le Bon et al., 2003), as well as efficient activation of naïve CD8⁺ T cells appears to depend on the expression of IP-10 and CXCR3 strongly enhanced in presence of type-I IFN (Ogasawara et al., 2002). A study of Santiago-raber showed that in vivo proliferation of T cells is dramatically reduced in NZB lupus-prone mice deficient for IFNAR1, as was the self-MHC/self peptide-mediated homeostatic proliferation of IFNAR1 KO T cells after transfer into the wt lymphopenic syngeneic host. Type-I IFNs are also known to exert antiproliferative and proapoptotic effects on T cells. These effects are associated to increased expression of cyclin-kinase inhibitors and pro-apoptotic molecules, and activation of pro-caspases. The final balance between IFN-α/β-induced proliferation and antiproliferation of T cell depends on the activation state of cells and on the inducibility of IFN-sensitive genes, that is reduced at the early phases of the immune response in activated cells as compared with T-naive precursors (Dondi et al., 2003). Type I IFNs influence immune responses also through the effects on NK cells. Type I IFNs have been shown to be critical for early NK cell responses to viral infections, such as lymphocytic choriomeningitis virus and murine cytomegalovirus, and for promoting NK cell cytotoxicity by a mechanism dependent on STAT1 (Lee et al., 2000; Biron et al., 1999). Moreover, type-I IFNs have been known to be central mediators of NK cell antitumor responses as playing a role in controlling NK cell-mediated antitumor responses in many experimental tumor models, including protection from methylcholanthrene-induced sarcomas, resistance to the NK cell-sensitive RMA-S tumor and cytokine immunotherapy of lung metastases (Swann et al., 2007).

1.4.4 pDCs/type-I IFN in autoimmunity

Given the pleiotropic effects of IFN-α/β in the innate and adaptive immune system, it is not surprising that these cytokines play a key pathogenic role in several autoimmune diseases. Among
them, evidence linking type-I IFN with the pathogenesis of lupus, psoriasis and Sjogren’s syndrome
in humans and animal model is the most convincing.

Systemic Lupus Erythematosus (SLE)

Increased levels of IFN-α in the serum of lupus patients were first noted over 30 years ago
and subsequently confirmed in several studies (Hooks et al., 1979). Although other cytokines are
also increased in lupus sera, IFN-α levels best coincide with exacerbation of the disease. It has been
largely shown that IFN-α treatment for a variety of conditions, including chronic viral infections
and cancer, also exacerbates or induce autoimmune manifestations like SLE. Moreover, convincing
evidence for a role of type-I IFN in lupus pathogenesis was provided by the finding that sera of
lupus patients, in particular during the active phase of the disease, induced in vitro maturation of
normal peripheral blood monocytes into DCs capable of efficiently capturing and presenting
antigens, and the active factor for this effect was IFN-α (Blanco et al., 2001). In addition,
miRNA studies with peripheral blood mononuclear cells of lupus patients showed increased
expression of IFN-α-induced genes (“interferon signature”) that correlated with disease severity
(Baechler et al., 2003; Bennett et al., 2003) in both early and late stages of the disease (Kirou et al.,
2003; Peterson et al., 2004).

Studies of spontaneous animal models of lupus have made it possible to directly demonstrate
the role of IFN-α/β in this disease. Evidences of acceleration of disease severity and onset by IFN-
α/β or its inducers in lupus-prone New Zealand Black (NZB) mice was reinforced by findings of
Theofilopoulos’s group that showed a significant amelioration of lupus disease in IFNAR1-
deficient NZB mice (Santiago-Raber et al., 2003). Homozygous and to a lesser extent heterozygous
deficiency of Ifnar1 gene led to reduction of many disease manifestations, including levels of IgG
anti-dsDNA autoantibodies, hemolytic anemia, glomerulonephritis, and mortality. The lupus-
promoting effects mediated by type-I IFN involved many immunocyte subsets, consistent with the
pleiotropic effects of type-I IFN. Numbers of B1 and conventional B2 subsets as well as numbers of
spontaneous antibody- and autoantibody-secreting cells were decreased in IFN-α/β-deficient NZB
mice. Significant decrease in the number of T cells, and in particular in the CD8+ subset, was also
observed in these mice, where reduced in vivo proliferation of IFN-α/βR-KO CD8+ T cells was
observed by BrDU analysis as well as during lymphopenia-induced homeostatic expansion.
Importantly, lack of IFN-α/β signaling in NZB mouse was found to have a major role on the
maturation and function of DCs in response of exogenous IFN-α. Another group reported a
decrease in glomerular immune complex deposits and lymphoadenopathy in C57BL/6-Faslox mice.
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knock-out for the *Ifnar1* gene (Braun et al., 2003). These animals showed lower IgG levels, reduction in activated and IgG-secreting B cells and B220+ T cells, proving direct evidence of the pivotal role of type-I IFN also in a different genetic background. Surprisingly, Hron and Peng observed the opposite effect in lupus-prone MRL-Fas<sup>lpr</sup> mice, with increased lymphoproliferation, autoantibody production and late-stage organ pathology in IFN-α/βR-KO animals, suggesting that effects of type-I IFNs are thus contextual and depending on specific genetic predispositions (Hron and Peng, 2004).

Overall, type-I IFN signaling appears to be crucial in switching on pathways that lead to pathogenicity in lupus-predisposed backgrounds. It has not been established, however, whether this switch is turned on in lupus because of a primary defect, such as hyper-production or hyper-responsiveness to stimuli, or because of a secondary abnormal condition, such as a viral or bacterial trigger, or increased levels of apoptotic cells and autoantigen-autoantibody immune complexes. What has been largely demonstrated is that in patients with SLE pDCs secrete IFN-α/β in response to DNA/RNA/immunocomplexes deriving from apoptotic/necrotic material that are internalized through FcγRIIa receptors and chronically stimulate TLR7 and TLR9 (Ronnblom et al., 2006). In addition, a very recent study of Gilliet’s group showed that complexes composed of antimicrobial peptides and self-DNA produced by activated neutrophils in the form of web-like structures, known as neutrophil extracellular traps (NETs), also play a critical role in pDC activation by chronic triggering of TLR9 (Lande et al., 2011). Based on these evidences, it has been, therefore, proposed that the effector stage of lupus-associated systemic autoimmunity is a process in which TLR-dependent induction of IFN-α/β by apoptotic materials and nucleic acids is a central event. In these models the initiation phase is mediated by FcγR-mediated uptake of IC formed with nucleic acid-containing macromolecules derived from late apoptotic/necrotic cells by pDCs. Increased type-I IFN production then cause cDC activation, leading to high expression of MHC-I and co-stimulatory molecules. Production of BLyS and APRIL by cDCs is also induced and, together with T cell help and other cytokines, like IL-6, IL-10 and IL-15, leads B cell differentiation to plasma cells and autoantibody production. Autoantibodies and/or immune complexes bind to cell surfaces and tissue and cause systemic autoimmune disease.

Similar mechanism may also cause organ-specific autoimmune diseases, but tissue damage and availability of previously sequestered self-antigens in lymph nodes is required for engagement of non tolerant cytotoxic CD8<sup>+</sup> T cells. Presentation of the released tissue-specific self-antigens by B cells to helper CD4<sup>+</sup> T cells may also occur, leading to autoantibody production. In both systemic
and organ-specific autoimmune diseases, IFN-α/β may also directly promote survival and proliferation of self-reactive T and B cells.

Psoriasis

Psoriasis is a chronic condition characterized by dermal infiltration of inflammatory cells, among which are T and B-lymphocytes producing Th1 cytokines. Recent findings have shown that pDCs also infiltrate psoriatic lesions during disease development and secrete large amounts of type-I IFN. In their initial observations, Nestle et colleagues (Nestle et al., 2005) found a strong IFN signature in primary psoriatic plaques, in the absence of detectable levels of IFN-α. IFN-α expression was, indeed, detected early and transiently during the development of the psoriatic phenotype whereas its effect persisted until lesion chronicization, as demonstrated in mice engrafted with pre-psoriatic skin. Xenograft models of human psoriasis allowed also to demonstrate that blocking IFN-α signaling or preventing pDCs production of IFN-α delayed the T-cell-dependent development of psoriasis, highlighting the important and necessary role of pDCs and pDCs-derived IFN-α in recruitment of inflammatory cells during initiation of psoriatic inflammatory reactions. Importantly, several groups have recently shown that pDCs infiltrating psoriatic skin can be activated to produce massive amounts of type-I IFN in response to extracellular self-DNA fragments. This process requires the coupling of self-DNA to the endogenous antimicrobial peptide LL-37, that is usually overexpressed in psoriatic skin (Frohm et al., 1997). LL-37 breaks tolerance.
to self-DNA by forming condensed structures that are sequestred in endosomal compartments where they trigger type-I IFN production through TLR9 (Lande et al., 2007).

Sjogren’s syndrome

Sjogren’s syndrome (SS) is a chronic slowly progressing autoimmune disease characterized by periductal lymphocytic infiltration of the exocrine glands, mainly the lacrimal and salivary glands, resulting in loss of their secretory function and subsequent dry eyes and mouth. Systemic manifestations are rather common, these are either related to the presence of periepithelial lymphocytic infiltrations of lung, liver, or kidney or to immune-complex mediated disease with cutaneous vasculitis, peripheral neuropathy, glomerulonephritis, and low C4 levels. Recent advances have revealed a major role for activation of the type-I IFN pathway in the pathogenesis of the syndrome, as evidenced by the increased circulating type-I IFN activity and an IFN signature in peripheral blood mononuclear cells and minor salivary gland (MSG) biopsies from SS patients (Zheng et al., 2009). Polymorphisms in genes involved in the IFNα pathway, such as IRF5 and STAT4, have been found to be associated with disease susceptibility (Nordmark et al., 2009), and while the initial triggers of the innate immune response in SS remain obscure, preliminary evidence supports the role of abnormally expressed retroelements as potential triggers of type I IFN activation in SS, possibly through TLR-dependent pathways (Mavragani et al., 2007).

Other autoimmune diseases

Detrimental effects of IFN-α/β have also been reported in several other autoimmune diseases, e.g. insulin-dependent diabetes mellitus (IDDM, type 1 diabetes), rheumatoid arthritis (RA), and autoimmune hemolytic anemia. In a study by Li et al. (Li et al., 2008), pDCs-derived type I IFN was implicated in the initiation of type I diabetes in non-obese diabetic (NOD) mice. This study demonstrated that pDCa are present in pancreatic lymph nodes and secrete IFN-α when pancreatic β-cell death is to peak, suggesting that pDCs are stimulated by apoptotic β-cell debris containing ssRNA and dsDNA that bind to TLR7 and TLR9, respectively. Accordingly, the authors found that blocking IFNAR early resulted in increased number of immature cDCs in pancreatic LNs, enhanced production of IL-4 and IL-10 by CD4+ T cells, and delayed beginning of disease in NOD mice.

Role of type I IFN in RA is controversial. One one hand, studies reported enhanced levels of IFN-α in synovial fluid and an increased expression of type-I IFN-inducible genes in peripheral blood cells
of a subgroup of patients who also expressed high circulating anti-cyclic citrullinated peptide antibody levels, the auto-antibodies associated with more destructive RA (Hooks et al., 1979; van der Pouw Kraan et al., 2007). On the other hand, it was reported that IFN-β inhibited collagen-induced arthritis in mice (van Holten et al., 2004) and had some beneficial effects in RA (Tak et al., 1999). Lastly, autoimmune hemolytic anemia was found to be associated to treatment with IFN-α for unrelated disorders (Andriani et al., 1996), and, as discussed earlier, it was found significantly reduced in IFNAR1-deficient NZB mice.

Demarcation of the immune system into innate and adaptive arms and recognition that endosomal TLRs sense nucleic acids have helped the understanding of how immune responses can be initiated. These developments have had a major impact on the comprehension of pathogenesis of autoimmune disorders. Within this framework, TLR-dependent induction of IFN-α/β by apoptotic materials and nucleic acids appears to be a central pathogenic event. Future challenges are to identify specific defects associated with IFN-α/β production and signaling, abnormalities in the generation, disposal and sensing of endogenous stimuli, and interactions between these effector cytokines and other disease-predisposing genes.

1.5 Wiskott-Aldrich Syndrome (WAS)

The actin cytoskeleton plays a crucial role in a variety of important cellular processes required for normal immune function, including trafficking, intercellular interactions, endocytosis, cytokinesis, signal transduction, and maintenance of cell morphology. Recent studies have unveiled not only many of the components and mechanisms that control the actin dynamics but have also revealed important pathological consequences associated with genetic alterations as actin cytoskeletal regulatory genes. These findings have provided new insight into the role of cortical actin cytoskeletal regulation in a number of immune cell functions and have identified cytoskeletal regulatory proteins critical for normal immune system activity and susceptibility to immune deficiency and autoinflammatory disease. Wiskott-Aldrich Syndrome (WAS) is an amazing demonstration of the importance of a highly regulated actin polymerization to support immune cell function. WAS is associated with multiple hematopoietic cell defects, and derives from mutations in WASp, a protein with actin-polymerizing activity, providing an ideal model to investigate the role of altered actin cytoskeleton in immune pathology.
1.5.1 The cytoskeleton

The cytoskeleton includes a filamentous network that serves to maintain cell shape and to regulate and promote important cellular functions. It provides an essential scaffold for the localization and activation/inhibition of several cytoplasmic signaling molecules, including protein and lipid kinases, GTPases, phospholipases, and GTPases, as well as anchors for motor proteins, such as kinesin, dynein, and myosin, that are critical for intracellular transport and cell division (Chesarone et al., 2009). Finally, the ability of cells to sense and respond to extrinsic and intrinsic stimuli also relies on the ability of the cytoskeleton to undergo remodeling.

The basic structural elements of the cytoskeleton comprise three filamentous macromolecules, filamentous-actin (F-actin), microtubules, and intermediate filaments. Actin filaments are composed of two actin chain polymers and play roles in maintaining cell morphology, assembly membrane protrusions, endocytosis, cytokinesis, and forming cell-to-cell or cell-to-matrix junctions.

Microtubules are cylindrical structures consisting of polymers of α- and β-tubulin that are organized by microtubule organizing center (MTOC), centrosomes and basal bodies. Microtubules are involved in the intracellular transport of organelles such as mitochondria, and in the formation of the mitotic spindle, and constitute flagella and cilia. Finally, the family of intermediate filaments includes about 70 different proteins subclassified into six types, of which vimentin is the most common. Intermediate filaments are the more stable components of the cytoskeleton and serve to maintain the cellular shape.

1.5.2 Actin filament assembly and disassembly

Actin filaments are formed by the unidirectional assembly of soluble monomeric globular-actin (G-actin) subunits and consist of pointed and opposite barbed ends, corresponding to the degrading or extending part of filaments. These polymers can either form linear bundles or a dendritic network. Linear actin filaments formation is catalyzed by the formin family and other types of nucleators like Cordon-Blue, Lmod, and Spire, in contrast, branched-chain F-actin dynamics are induced solely by the Arp2/3 complex, that starts the addition of actin polymers at a 70° angle to existing mother filaments. After nucleation, open barbed end of actin filaments continue to spontaneously extend until terminated by capping proteins like gelsolin or capping protein muscle Z-line (CAPZ). Capping activity is modulated by anti-capping proteins, such as ENA and vasodilator-stimulated phosphoprotein (VASP), which, by protecting the growing barbed
end from abundantly present capping proteins, promote continued filament extension (Chesarone et al., 2009). Nucleation-promoting factor (NPF) complexes play a crucial role in regulating Arp2/3 complex activation. These complexes, which require a WASp family or a Wiskott-Aldrich syndrome family verprolin-homologous protein (WAVE 1, 2, or 3) member, plus several other proteins, such as hematopoietic protein1 (Hem1), WASp interacting protein (WIP), and Abl interacting adaptor protein, activate Arp2/3 following the activation of WASp members by Cdc42-GTP or WAVE members by Rac (Snapper and Rosen, 1999). Disassembly of older actin filaments is mediated by actin-depolymerizing factor (ADF)/cofilin family of proteins that cut older, ADP-rich filaments. Cofilin is activated by dephosphorylation of Ser-3 by Slingshot phosphatase (Kurita et al., 2008) or chronophin (Gohla et al., 2005) and inhibited by phosphorylation of Ser-3 by LIM kinases (LIMK) (Edwards et al., 1999; Vicente-Manzanares and sanchez-Madrid, 2004). For branched chains, disassembly is also mediated by Coronin, which displaces the Arp2/3 complex from older branched point destabilizing the branched chain and by binding to Slingshot, promotes the dephosphorylation (activation) of cofilin at the same place (Solderling 2009).

![Figure 7. F-actin assembly and disassembly](image-url)

**Figure 7. F-actin assembly and disassembly.** Branched-chain F-actin dynamics: (1) Small GTPases, such as Cdc42, Rac1, and RhoA, are activated by extracellular stimuli. (2) In resting cells, WASp family of NPFs, WASp, and WAVE/SCAR remains in an inactive conformation. Binding of Cdc42 or Rac1 activates WASp or WAVE/SCAR, respectively. (3) These, in turn, bind actin and the Arp2/3 complex stabilizing the latter in its active conformation, which induces nucleation of new filaments at a 708 angle on existing mother filaments. (4) Barbed ends extend until they are terminated by capping proteins such as CAPZ and gelsolin. (5) Binding of anti-capping proteins, such as ENA and VASP, promotes filament extension by protecting barbed ends and by promoting the addition of actin monomer units bound to profilin. (6) HS1 (the leukocyte specific homologue of cortactin) binds to and stabilizes Arp2/3 complexes at branch points, which displaces WASp or WAVE/SCAR. (7) At the aging end of the F-actin network, Coronin and Slingshot replace the Arp2/3 complex at branched points leading to variable angles and instability of F-actin branches. (8) Cofilin and Wdr1 induce F-actin de-polymerization by severing and enhancing the off-rate of actin monomers. (9) Thymosin b4 (Thyb4) sequesters actin monomers (G-actin) and inhibits actin polymerization. (10) Cofilin is inhibited by LMIK. LIMK itself is activated by two different pathways, activation of ROCK (Rho-associated, coiled-coiled containing protein kinase) by RhoA and activation of PAK (p21-associated kinase) by Rac1 and Cdc42. (11) Cofilin is activated by Slingshot mediated dephosphorylation. Unbranched F-actin assembly. (12) Actin filaments are polymerized by formins such as mDia1 following activation of the specific formin by RhoA. Profilin catalyzes this process.
1.5.3 Immune cellular functions dependent on actin cytoskeletal dynamics

The actin cytoskeleton participates in many different cellular processes as it provide the basic framework for a wide array of subcellular structures. Several actin cytoskeletal regulators control biological responses that are associated to Arp2/3-mediated actin dynamics and are required for appropriate immune functions. These functions include membrane and cell motility, cell-cell interactions and endocytosis. Among the actin assembly-regulator factors, WASp and Neural-(N)-WASp are those best characterized.

Actin cytoskeletal structures in membranes and cell motility

Immune cell migration critically mediates tissue-specific cell trafficking and, therefore, the outcome of immune responses. During migration and tissue invasion, immunocytes utilize various structures for forward propulsion such as filopodia and podosomes. Filopodia are slight actin cytoskeletal projections whose formation requires both linear and branched-chained F-actin. Several actin cytoskeletal proteins regulators have been implicated in this process, including the small GTPase Cdc42 and N-WASp. N-WASp localizes to certain types of filopodium, however, filopodia still form in absence of N-WASp indicating a dispensable role of the protein in the assembly process (Snapper et al., 2001). Podosomes and invadopodia are protease-rich protrusions utilized during tissue invasion by macrophages, monocytes, and metastatic cancer cells that facilitate degradation of the extracellular matrix allowing cells to advance. Src family tyrosine kinases and N-WASp are required for podosome and invadopodium formation as expression of a dominant-negative mutant of N-WASp in Src-transformed cells was found to associate to inhibition of invadopodium formation and degradation of the extracellular matrix. In addition, the binding of the Src family substrate cortactin to N-WASp was shown to actively regulate this process by promoting N-WASp recruitment to the site where Arp2/3 is localized, so inducing its strong activation and podosome forward (Mizutani et al., 2002). Finally, Tsuboi showed that a complex of WASp with WASp interacting protein (WIP) is required for podosome formation in macrophages (Tsuboi 2007 a), whereas a study of Olivier and colleagues reported that a partial reduction in the levels of WASP was sufficient to inhibit development of podosomes in dendritic cells (Olivier et al., 2006). Very recently, it has been shown that WASp in concert with Hematopoietic lineage cell-specific protein 1 (HS-1) functions to promote correct podosome array organization by DCs (Dehring et al., 2011).
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*Cell-cell interactions*

Regulation of the actin cytoskeleton is also critical for intercellular interactions, particularly at the immune synapse between APCs and T cells, where a dense actin network is required to assure a tight cell adhesion. WASp, for example, plays a crucial role by controlling ex novo actin polymerization required to stabilize synapse formation and signaling. WASP deficiency associates with altered localized assembly of filamentous actin and impaired recruitment of several immunological synapse proteins in response to TCR stimulation (Snapper et al., 2005; Sasahara et al., 2002; Badour et al., 2003; Sims et al., 2007; Dupre et al., 2009). Moreover, our group and others, recently unveiled a role for WASp on the APC side of the immunological synapse for a long-lasting stable interaction and proper transmission of activating signals (Pulecio et al., 2008; Bouma et al., 2011).

*Endocytosis*

Endocytosis is an essential cell-surface membrane trafficking event that delivers soluble molecules, membrane components or receptors, with their associated ligands, to the endocytic pathway. There are at least two basic endocytosis mechanisms: clathrin-dependent endocytosis, in which receptors and their bound ligands are internalized by clathrin-coated pits, and clathrin-independent endocytosis (e.g. phagocytosis, macropinocytosis, caveolar endocytosis). In clathrin-dependent endocytosis, vesicle coats consist of clathrin and the heterotetramer Assembly Protein 2 (AP2), that links, directly or indirectly, receptors and clathrin. The pinching off of the clathrin-coated domain requires accessory proteins, such as epsin, which link receptors to coat components and contribute to the bending of the lipid bilayer (Ford et al., 2002). Finally, in both clathrin-dependent and clathrin-independent endocytosis, Dynamin is required for vesicle fission from the donor membrane. After fission of clathrin-coated vesicles (CCVs) from the plasma membrane, the clathrin coat is removed and recycled for another round of transport. Uncoated vesicles fuse together to form new endosomes, or fuse with pre-existing early endosomes (EE) (Maxfield and McGraw 2004). EE are entry portals, sorting stations and signaling platforms pivotal in directing molecules into the appropriate pathway. Degradative molecules are sorted into specific membrane domains and this process is followed by maturation with acidification and formation of compartments named multivesicular bodies (MVBs) (Stoorvogel et al., 1991; Futter et al., 1996). Finally, MVBs/late endosomes (LE) fuse with lysosomes where protein degradation and signal switch off occur. In APCs, MVBs can also fuse back to the cell surface as secretory exosomes.
Furthermore, recycling molecules are directly transported to the plasma membrane by vesicular transport or indirectly by recycling endosomes (RE) through large tubules (Maxfield and McGraw 2004). In endocytosis, actin polymerization seems to have important roles particularly in the step of vesicle fission and in subsequent vesicle trafficking inside the cell. Particularly, actin was shown to provide a motile force that supports the fission activity of dynamin GTPase (Roux et al., 2006), and promote endosome movement and morphology (ohashi et al., 2011 a). Among the actin-related proteins required for controlling endosomal actin reorganization, N-WASp/WASp has been described to regulate Arp2/3 activity at EEs ensuring appropriate endosomal motility and maturation toward the perinuclear region (Taunton et al., 2000; Chang et al., 2003).

1.5.4 Nucleation-promoting factors: the WASp family

The WASp family encompasses a group of evolutionarily conserved cytosolic proteins that share a C-terminal verprolin homology/central/connecting/acidic region (VCA) domain enabling each protein to associate with actin monomers and to bind and induce actin-nucleating activity of the Arp2/3 complex (Takenawa and Suetsugu, 2007). Among these proteins, WASp and the ubiquitously expressed neural-WASp (N-WASp) are distinguished by an N-terminal Ena/VASP homology 1 (EVH1) domain, the three WAVE/SCAR proteins (WAVEs 1, 2 and 3) by an N-terminal SCAR homology domain (SHD), and the recently identified WASH protein (Wiskott–Aldrich syndrome protein and Scar homology) by two N-terminal WASH homology domains (Linardopoulou et al., 2007).

WASp and N-WASp are approximately 500 amino acid cytosolic proteins comprised of an N-terminal EVH1 domain, a GTPase-binding domain (GBD) that contains a lysine-rich basic region (BR) and a Cdc42/Rac1 interactive binding (CRIB) motif, followed by a central proline-enriched region, and a C-terminal VCA domain. The VCA domain mediates WASp/N-WASp binding to the Arp2/3 complex, an interaction that induces Arp2/3 conformational changes which allow actin-nucleating activity. The association of WASp/N-WASp to the Arp2/3 complex is influenced by the CRIB motif, which mediates physical association with activated (GTP-bound) Cdc42. This interaction promotes activation of WASp/N-WASp, releasing the proteins from an autoinhibitory structural constraints given by intramolecular interactions between the CRIB and VCA domains (Rohatgi et al., 1999; Kim et al., 2000). Activation of N-WASp via binding of its CRIB domain to other Rho GTPases and its BR domain to phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P2) has also been reported (Tomasevic and al., 2007). Importantly, expression of GBD-deleted forms of WASp or N-WASp does not impede antigen receptor-triggered actin polymerization and
proliferation in T cells and vesicular trafficking in neurons, functions mediated by WASp and N-WASp, respectively (Kato et al., 1999; Benesch et al., 2002). Thus, GBD binding to activated Cdc42 promotes but may not be an absolute requirement for inhibition of constitutively inactivated WASp/N-WASp. In contrast, mutation of the major tyrosine phosphorylation site on WASp (Y291) or N-WASp (Y256) or dephosphorylation of this site by the PTP-PEST tyrosine phosphatase abrogates the inducible coupling of these proteins to actin polymerization (Badour et al., 2004). Moreover, interactions of these proteins with various adapters and dimerization of active WASp species have also been shown to enhance VCA domain effects on Arp2/3 activity (Padrick et al., 2008). Thus, while the CRIB motif clearly enables WASp/N-WASp interactions with activated Cdc42, WASp/N-WASp activity seems to be regulated by a multiplicity of mechanisms and effector interactions (Prehoda et al., 2000). As for the GBD, also the WASp/N-WASp N-terminal EVH1 plays an important role in regulating WASp/N-WASp activity. Initially identified in another conserved family of cytoskeletal regulators (the Ena/ VASP proteins) (Bachmann et al., 1999), the EVH1 domain has been shown to interact via an F/W/Y/LPPP motif with proline-rich regions on other effectors, (Fedorov et al., 1999). Among the EVH1 domain-mediated interactions, the best characterized from a functional perspective is the one involving the WASp-interacting protein (WIP). WIP appears to bind WASp constitutively via a proline-rich motif and to be released from this interaction following protein kinase C h (PKC-h)-mediated WIP phosphorylation (Volkman et al., 2002; Sasahara et al., 2002). WIP binding stabilizes the WASp/N-WASp molecules and also suppresses their capacity for activating Arp2/3 activity (Krzewski et al., 2006; sawa and Takenawa, 2006). These effects of WIP may be regulated by its interaction with another adapter, Toca-1 (transducer of Cdc42-dependent actin assembly), a member of the pombe Cdc15 homology (PCH) family that appears to promote N-WASp activation by direct interaction with Cdc42-GTP and WIP-bound N-WASp (Ho et al., 2004). The precise roles for Toca-1, however, in regulating WASp/N-WASp activity require further investigation as do the roles of two WIP-related proteins, CR16 and WICH (WIP/CR16 homologous protein), which also bind WASp/N-WASp via the EVH1 domain (Kato et al., 2002; Ho et al., 2001) WASp/N-WASp functions also appear highly dependent on the proline-rich region, a segment of about 200 residues between the GBD and VCA domains. Residues across this region interact with SH3 domains of Fyn and Hck protein tyrosine kinases, phosphoinositol 3-kinase (PI3K) and phospholipase Cc (PLCc)( Badour et al., 2004; Cory et al., 2002; Suetsugu et al., 2002; Banin et al., 1996) and many adapters, including Nck, Grb2, PSTPIP1, intersectin 2, and WISH (Fukuoka et al., 2001; Rivero-lezcano et al., 1995; Banin et al., 1996; Tian et al., 2000; McGavin et al., 2001; Badour et al., 2003; She et al., 1997; Carlier et al., 2000). Many of these adapter interactions enhance WASp/N-WASp effects on Arp2/3 activity (Padrick et al.,
2008) and redirect WASp/N-WASp subcellular localization so as to enable diverse cellular processes, including synapse formation (PSTPIP1 and Nck), endocytosis and vesicular trafficking (intersectin2 and SNX9), and microtubule dynamics (CIP4, FBD17) (Badour et al., 2004; Badour et al., 2003; Cannon et al., 2001; Yarar et al., 2007). Interactions of LPPP motifs within this region with the VASP have also been shown to promote N-WASp activity by mediating its association with another cytoskeletal regulator, profilin (Suetsugu et al., 1998; Yang et al., 2000; Castellano et al., 2001, while Fyn and Hck induce WASp and N-WASp activation by inducing their tyrosine phosphorylation (Badour et al., 2004; Cory et al., 2002).

1.5.5 Wiskott-Aldrich syndrome, general traits

Wiskott-Aldrich syndrome (WAS, OMIM 301000) is a complex and severe X-linked disorder caused by mutations in WAS gene (WASp), a hematopoietic specific regulator of actin
nucleation. WASp deficiency is the most common Mendelian-inherited human disease of an actin regulatory protein and, depending on the severity of the WASp defect, manifestations can range from the full-blown WAS to X-linked thrombocytopenia (XLT). WASp patients typically develop thrombocytopenia, a high incidence of eczema (about 80%), and immunodeficiency, the latter of which is associated with an increased risk of infection and malignancy. Despite the immunodeficiency, there is paradoxically a high incidence (40-72%) of certain autoimmune diseases, most commonly autoimmune hemolytic anemia, followed by vasculitis, arthritis, renal disease, inflammatory bowel disease and neutropenia (Ochs and Trasher, 2006; Bosticardo et al., 2009). Mutation impairing but not abolishing WASp expression can cause XLT. This disease can be chronic or intermittent, and is considered an attenuated form of WAS because it is characterized by low platelet counts with minimal or no immunodeficiency. Recently, gain-of-function mutations in the WAS gene, that give rise to a constitutively active protein, are found to cause a distinct pathology, the X-linked neutropenia. X-linked neutropenia is characterized by low neutrophil number and predisposition to myelodysplasia in the absence of thrombocytopenia and T-cell immunodeficiency (Notarangelo et al., 2002; Ancliff et al., 2006).

The wide spectrum of clinical manifestations highlights the complex role of WASp in various cellular mechanisms. WASp deficiency is associated with impaired development, migration, activation, and/or adhesion of platelets, lymphocytes, neutrophils, mast-cells, and dendritic cells, of which T cells are the most severely affected.

WASp deficient mice (WKO mice) exhibit similar defects in hematopoietic cells as WAS patients, thereby providing a helpful model to study the pathophysiology of the syndrome. However, the clinical manifestations of WKO mice differ somewhat, in that they consist primarily of immunodeficiency, thrombocytopenia and a late onset inflammatory bowel disease (Snapper et al., 1998; Zhang et al., 1999). WKO mice have a high incidence of antinuclear antibodies with high levels of anti-DNA (Humblet-Baron et al., 2007) (also discussed later), whereas only a single case of SLE has been so far reported in WAS patients (Monteferrante et al., 2009) (also discussed later).

1.5.6 Cellular defects in WAS

WAS arises from mutations in the gene that codes for WASp. Extensive analysis of WASp activation, regulation and function in T lymphocytes, have contributed to the understanding of the molecular basis of the immunodeficiency in WAS patients. However, it is increasingly evident that a general impairment of hematopoietic cell functions contributes to the pathogenesis of the disease.
T-cell defects in WAS

T-cell defects, hampering both effector and helper functions, play a crucial role in WAS-associated immunodeficiency. WASp plays a key role in T-cell activation and actin cytoskeleton remodeling after the engagement of the TCR (Gallego et al., 1997; Snapper et al., 1999), and the co-stimulatory molecules CD28 (Badour et al., 2007) and CD2 (Badour et al., 2003). T-cell activation is regulated by the formation of the immunologic synapse (IS), a polarized cluster of TCR, co-stimulatory molecules, and integrins at the T-cell-APC interface. It has been shown that in absence of WASp, IS can be formed only after strong TCR stimulation (Cannon et al., 2004). In addition, lipid raft dynamics, crucial for the movements of the IS molecules to the plasma membrane, are compromised during IS formation (Dupre et al., 2002) and IS stability (McGavin et al., 2001). Another level of regulation of T-cell activation is obtained through the internalization of the TCR and CD28 co-stimulatory molecule after specific engagement, functions that are defective in WASp-deficient cells (Badour et al., 2007; Haddad et al., 2001). As a consequence of impaired signaling through the TCR and co-stimulatory molecules, T cells from WAS patients and WAS knock-out (WKO) mice show defective proliferation as well as impaired secretion of IL-2 and Th1 cytokines (Snapper et al., 1998; Badour et al., 2007). In addition to its role in T-cell activation, WASp is also critical for T-cell chemotaxis in vitro in response to stromal cell-derived factor-1α (Haddad et al., 2001) and in vivo homing to secondary lymphoid organs (Gallego et al., 2006). A reduction in the number of circulating naïve CD4+ and CD8+ T cells is also present in WAS patients, especially at a young age, contributing to the immunodeficiency characterizing the disease (Park et al., 2004).

Role of WASp in B cells

The description of B-cell anomalies was mainly focused on the defective cytoskeletal-dependent processes of WKO cells leading to decreased migratory ability, adhesion, and formation of long protrusions (Westerberg et al., 2001; Westerberg et al., 2005; Vermi et al., 1999). Consequently, the decreased motility of WKO B cells has been considered the main cause of findings showing diminished B-cell number in peripheral blood and secondary lymphoid organs of WAS patients (Park et al., 2004) and later confirmed in the WKO murine model (Westerberg et al., 2001). Recent studies have shown an early deficit of B cells starting from infancy and indicating a defective cellular output (Park et al., 2004). The subsequent characterization of B-cell defect in WAS patients led to identification of a phenotypic perturbation with regard to complement receptors and CD27 (Park et al., 2005). Indeed, B cells from WAS patients show a reduced
expression of CD21/CD35 receptors that may be responsible for impaired capability of antigen capture and presentation. In addition, WAS patients present a reduced amount of CD27+ postgerminal center B cells, indicating a defective differentiation, despite normal amount of class switching (Park et al., 2005). Recently, studies unveiled a role for WASp in peripheral homeostasis of mature B-cell subsets. WASp seems to be dispensable for early B-cell development, whereas its deficiency is detrimental for completion of B-cell maturation, starting from transitional stage and affecting in particular splenic marginal zone and peritoneal B1a cells. This phenotype appears to be caused by a defective homeostasis and/or retention of mature B cells, rather than increased apoptosis of WKO B cells (Westerberg et al., 2008; Meyer-Bahlburg et al., 2008). Finally, recent evidence has begun to emerge demonstrating a role for B cell-intrinsic mechanisms in WAS-associated autoimmunity (Becker-Herman et al., 2011; Recher et al., 2012) (also discussed later).

**Role of WASp in dendritic cells**

The effects of mutations of WAS gene in DCs result in severe alterations in migration, antigen presentation, cell adhesion, and T-cell priming. Many groups have showed the role of WASp in the formation of podosomes in DCs. In human DCs, the absence of WASP results in the complete abrogation of podosomes, while in the murine system, DCs from WASP null mice are largely devoid of podosomes, showing disorganized clusters of F-actin dots reminiscent of poorly ordered podosomal structures. Importantly, podosome defect resulted in alteration of β2 integrin localization, which remained dispersed with consequent decreased adhesion to ICAM-1, a ligand for β2 integrin (Burns et al., 2004; Calle et al., 2004). These findings suggest that WASp plays a crucial role in providing a platform for integrin organization at the cell membrane. Moreover, WKO DCs failed to form lamellipodia, resulting in a defective chemotactic response to different chemoattractants, such as FMLP, MCP-1 (Badolato et al., 1998; Zicha et al., 1998). In particular, the migratory response of WAS mutant DCs toward the CCR7 ligands, CCL21 and CCL19, which are highly expressed on high endothelial venules and lymphatic endothelium, is severely decreased (de Noronha et al., 2005; Snapper et al., 2005). Similarly, chemotaxis of WKO immature DCs to CCL3, crucial for mobilization to inflammatory sites, is significantly reduced (Snapper et al., 2005). Additionally, WKO DCs do not spread correctly and fail to form stable leading edge, resulting in defective directional migration and inefficient homing into lymph nodes (Bouma et al., 2007). Finally, our group and others demonstrated that, besides the impaired motility, WKO DC show defective interaction with and activation of CD4+ and CD8+ T cells in lymph nodes (Bouma et al., 2011; Pulecio et al., 2008). Indeed, we showed that, especially at low antigen doses, the frequency
and duration of WKO DCs-T cell contacts, in vitro and in vivo, was reduced and CD8\(^+\) T cell priming resulted inefficient, remarking the relevance of WASp in DCs during the first phases of adaptive immune response (Pulecio et al., 2008).

1.5.7 Autoimmune manifestations in WAS

WAS-associated autoimmune complications are frequently observed (40-72%) (Sullivan et al., 1994; Dupuis-Girod et al., 2003). The most common manifestations are autoimmune hemolytic anemia, cutaneous vasculitis, arthritis, and nephropathy. Other less frequent autoimmune diseases include neutropenia, dermatomyositis, recurrent angioedema, uveitis, and cerebral vasculitis. Patients frequently have multiple autoimmune manifestations at the same time (Dupuis-Girod et al., 2003). The incidence of autoimmune diseases in patients with XLT is generally less frequent than in patient with fully WAS. However, a study conducted on Japanese patients reported that autoimmune disorders are equally frequent in patients with a low clinical score (representing XLT) as in those with a high clinical score (representing WAS). Development of autoimmunity can have a prognostic value. It has been reported that WAS patients who develop autoimmune hemolytic anemia or autoimmune thrombocytopenia early (<180 days) after splenectomy have a poor prognosis (Dupuis-Girod et al., 2003). Moreover, autoimmunity is associated with a higher risk of a later development of tumors and an increased risk of mortality (Sullivan et al., 1994). Recently, a study of Monteferrante et al. documented one patient in whom SLE developed on a WAS gene-mutated background (Monteferrante et al., 2009). SLE was diagnosed because the patient fulfilled four criteria according to the American Rheumatism Association (ARA) classification for definite SLE (Hochberg 1997), that are arthritis, proteinuria and hematuria, positive antinuclear antibody testing, and haemolytic anaemia with lymphopenia. The patient presented a mutation in the EVH1 domain of WAS gene, with a marked reduced expression of the specific messenger.

Until now the mechanisms of WAS-associated autoimmunity remain the less understood aspect in the pathophysiology of this disease. It has been proposed that autoimmunity could be the result of a bystander tissue damage originating from the chronic inflammatory state that is established after incomplete pathogen clearance (Arkwright et al., 2002). Recently, several groups have described that, in absence of WASp, CD4\(^+\) CD25\(^-\) FOXP3\(^+\) regulatory T cells (Tregs), crucial to prevent autoimmune diseases by down-modulating immune response to allergens, pathogens, and cancer cells, show defective localization and function (Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007). WASp seems to be dispensable for thymic development and steady state distribution of natural regulatory Treg to the periphery, although a selective advantage for
WASp expressing nTreg cells has been described in the competitive setting of WASP+/- mice (Westerberg et al., 2008). Despite a relatively normal number of CD4+ CD25+ FOXP3+ cells in spleen of WKO mice and in peripheral blood of patients, nTreg showed a marked defect in suppressor activity. In vitro experiments showed that nTregs from WKO mice had a significantly reduced capacity to suppress wt effector T-cell proliferation. Similarly, in WAS patients nTreg cells displayed poor suppressor activity toward normal and WKO effector T cells in vitro (Marangoni et al., 2007). Besides defective in vitro nTreg suppression activity in the WAS murine model and in patients, impaired in vivo suppressive activity of WKO nTreg was also demonstrated. Maillard et colleagues reported a defective in vivo suppressor activity of WKO nTreg toward colitis induced by transfer of wt CD45RBhi cells. In line with these findings, spontaneous genetic reversion of WAS mutations in nTregs of a WAS patient correlated with amelioration of autoimmune manifestations (Humblet-Baron et al., 2007). Importantly, Marangoni et al. showed that WKO nTregs transferred in wt hosts were unable to reach LNs draining the site of OVA immunization and consequently modulate a response to OVA. This study suggested that besides an intrinsic dysfunction in mediating suppression, WKO nTregs may also display in vivo impaired migration, survival and/or proliferation.

Finally, a very recent study of Rawlings group showed that mutations of the WAS gene in B cells associate with hyper-responsiveness to BCR and TLR triggering. Interestingly, mixed bone marrow chimeras, in which only the B cell lineage lacked WASp, exhibited severe autoimmunity, as compared to WKO mice, unveiling for the first time a B cell-intrinsic mechanism of loss of tolerance sufficient to drive autoimmune disease in a contest of normal T cell function (Becker-Herman et al., 2011). Almost in parallel, Recher et colleagues generated a mouse with selective deficiency of WASp in the B cell lineage. In this way they excluded the possibility that mixed chimerism in non-B lineages, irradiation-induced load of apoptotic bodies and homeostatic B cell proliferation may contribute to autoimmunity. B-depleted mice showed signs of B cell dysregulation, as indicated by an increase in serum IgM levels, expansion of germinal center B cells and plasma cells, and elevated autoantibody production. This was accompanied by hyperproliferation of WASp-deficient follicular and germinal center B cells in heterozygous counterpart in vivo and excessive differentiation of WASp-deficient B cells into class-switched plasmablasts in vitro, strongly supporting a contribution of B cell-intrinsic mechanisms in WAS-associated autoimmunity (Recher et al., 2012).
In spite the importance of WASp in DCs during the first phases of adaptive immune responses, there are still no data relative to mechanisms by which perturbation of actin dynamics in DCs may promote autoimmunity.
AIMS

Pathogenesis of autoimmune complications in WAS are still poorly understood. In recent years, several studies have documented impairment in the T and B cell compartments providing interesting hints to understand loss of tolerance. However, an integrated view of how the different cell compartments cooperate in abolishing peripheral tolerance has not been yet defined. Of note, a mechanistic link between actin and development of autoimmunity is missing. In type-I IFN diseases aberrant production of IFN-α by pDCs was shown to be an early upstream event preceding autoimmune manifestations. Based on the clinical features of WAS, we postulated that pDCs/IFN-α axis may have a role in WAS-associated autoimmunity.

During my PhD, I have undertaken the analysis of pDCs and IFN-α production in a mouse model of the disease evaluating the possible contribution of altered pDCs function in disease pathogenesis. For that purpose, my thesis work had three major objectives:

1) The analysis of the pDCs compartment and the pDCs functionality in response to TLR triggering.

2) The analysis of the mechanism by which WASp may cause altered IFN-α production.

3) The validation in human WAS patients
2  MATERIALS AND METHODS
Mice

WASp\textsuperscript{+/−} mice on a C57BL/6 (CD45.2) genetic background were a gift from S. Snapper (Massachusetts General Hospital, Boston, MA). Mice were bred and maintained in sterile isolators. \textit{In vivo} experiments were performed using WASP\textsuperscript{+/−} homozygous female or WASP\textsuperscript{−/−} male as WASp KO mice and wild type (WT) littermate as control. OVA-specific, MHC class I restricted and MHC class II, TCR transgenic OT I and OT II mice were purchased from the Jackson ImmunoResearch Laboratories. CD45.1 congenic C57BL/6 (a gift from P. Guermonprez, Institut Curie, Paris, France) were bred to OT-I mice to obtain OT-I/CD45.1. 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

BM-derived pDCs were differentiated in vitro from the bone marrow of WT or WASp KO mice using FLT3 ligand (FLT3-L). DCs were used for experiments between days 6 and 8. BM-pDC were obtained by positive selection using B220\textsuperscript{+} microbeads (Miltenyi Biotec). For isolation of pDCs 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 pDCs were enriched from total spleen cells by negative selection using CD19\textsuperscript{+} and CD3\textsuperscript{+} microbeads followed by positive selection with PDCA-1\textsuperscript{+} microbeads (Miltenyi Biotec). In some experiments CD11c\textsuperscript{+}/PDCA-1\textsuperscript{+}/B220\textsuperscript{+} cells were isolated from B- and T-cells-depleted spleen cells or total lymph node cell suspensions using a FACS Aria III cell sorter (BD Bioscences). Purity was higher that 95%. OT-I and OT-II cells were isolated from total lymph nodes suspension by negative selection using MACS isolation kit.

TLR agonists

The CpG-A (1585) and CpG-B (1826) oligonucleotides were from InVivoGen, San Diego, CA. The Cy5-labeled CpG-A was obtained from Sigma-Aldrich. The lipopolysaccharide (\textit{Escherichia coli} O55:B5) was from Enzo Life Sciences, Inc.

Bacterial strains

\textit{Salmonella enterica serovar Thypimurium} strain 3261 ATK (\textit{InvA}) expressing ovalbumin is a gift from Dr. M. Rescigno (IEO, Milano). \textit{Escherichia Coli} strain DH5\textalpha{} (\textit{InvA}) expressing ovalbumin was kindly provided by Dr. A. Savina (Institut Curie, Paris).
FACS analysis

The following Abs for FACS analysis were purchased from BioLegend: PE-conjugated Sca-1, PE-Cy7-, FITC- and PE-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, FITC- and APC-conjugated anti-PDCA-1, PE conjugated Siglec-H, PE-Cy5-conjugated anti-CD19, FITC- and APC-conjugated anti-CD3. Biotinylated Anti-mouse CD69 antibody was purchased from BD PharMingen, and Alexa Fluor 488 streptavidin from Life Technologies, Invitrogen. Anti-IFN-α RMMA-1 was from PBL Biomedical Laboratories, Inc. FITC-conjugated anti-WASp was purchased from Santa Cruz Biotechnology, Inc.

Stained cells were acquired with a FACS Aria III flow cytometer and analyzed with FLOWJO software (version 4.5.4; Tree Star Inc.). Isotype controls were performed with corresponding rat Ig. For detection of intracellular IFN-α, 10 μg/ml Brefeldin A (Sigma-Aldrich) was added during all the phases of DC enrichment procedure and staining. Cells were enriched from total spleen cells by depletion of B and T cells, and stained for extracellular markers using antibodies to CD11c, B220 and PDCA-1. Cells were then processed with the Fix & Perm kit (BD Bioscences) according to the manufacturer’s instructions and then stained over night at 4°C with an anti-IFN-α antibody.

In vivo treatment

Mice were injected in the tail vein with CpG-A complexed with the cationic lipid 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP). For preparation of CpG-A/DOTAP mixture, 10 μg CpG-A resolved in 50 μl of PBS were incubated with 30 μl (30 μg) of DOTAP Liposomal Transfection Reagent (Roche) in a polystyrene tube containing 70 μl of PBS for 15 min at room temperature. The CpG-A/DOTAP mixture (150 μl) was then injected. Sera were collected 2 and 6 h after the CpG-A/DOTAP injection and analyzed for the content of IFN-α, IL-6 and IL-12p40. For the detection of intracellular IFN-α, spleens are collected 45 min or 2 hrs and 30 min after the injection. Cytokine ELISA kits were purchased from PBL Biomedical Laboratories (IFN-α) and Thermo Fischer Scientific (IL-12p40 and IL-6).

Mice were also injected with LPS, CpG-B, and Salmonella typhimurium. CpG-B (25 μg), LPS (100 ng), or S. typhimurium (1,2 x 10^6 CFU) resolved in 100 μl of PBS were injected in the tail vein. After 4 hrs, mice were sacrificed and the spleens collected. After tissue homogenization and digestion, splenocytes were stained for the analysis of maturation markers by FACS. Sera were also collected after the sacrifice for the analysis of IL-12p40 and IL-6 content.
BrdU incorporation and cell death

For continuous in vivo bromodeoxyuridine (BrdU) labeling, mice were fed BrdU (1 mg/mL from Sigma-Aldrich) in 10% sucrose-supplemented drinking water for 7 days and then sacrificed. BrdU incorporation in spleen, lymph node and bone marrow cells was determined using the FITC BrdU flow kit (BD Biosciences). For cell death analysis, splenocytes stained with antibodies to CD11c, B220 and PDCA-1 were labeled with FITC-conjugated annexin V and 7-amino-actinomycin D (7-AAD; BD Biosciences). Dead cells were defined as 7-AAD\(^+\) cells and apoptotic cells as 7-AAD\(^-\) annexin V\(^+\).

RNA extraction and real-time polymerase chain reaction

Total RNA from isolated endogenous pDCs of pooled mice, or from bone-marrow-derived pDCs, was prepared with TRIZol (Invitrogen) and treated with DNase I (Fermentas) according to the manufacturer's protocol. cDNA was synthesized with random hexamers and M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR analysis was performed with the CFX96 Touch™ Real-Time PCR Detection System and iQ SYBR green Supermix (Bio-Rad Laboratories). \(\beta\)-actin mRNA was used for normalization of Ifna4, Il6 and Tlr9 mRNA expression. Primer sequences were as follows:

**IFN-\(\alpha\)4:** forward, 5′-CCTGTGTGATGCAGGAACC-3′ and
revers, 5′-TCACCTCCCAGGCACAGA-3′;

**IL-6:** forward, 5′-GAGGATACCACCTCCCAACAGA-3′ and
revers, 5′-AAGTGCATCATCGTTGTTCAT -3′;

**TLR9:** forward, 5′-CCAGACGCTCTTGGAGGGAACC-3′ and
revers, 5′-GTTATAGAAGTGGCGGTTGT -3′;

**\(\beta\)-actin:** forward, 5′-CAGAAGGAGATTACTGCTCTCCACAGAACC-3′ and
revers, 5′-GGAGGCACCCGATCCACACA-3′.

Thermal cycle conditions were the following: 95°C for 3 min, 43 cycles of 95°C for 10 s, and 60°C for 30 s. Each sample was analyzed in triplicates. \(\Delta CT\) were obtained by normalizing target genes to the housekeeping gene. The relative expression of target genes was defined as \(2\Delta CT \times 10^3\).
Silencing of WASp

ONE-TARGET plus siRNA targeting WASp and ONE-TARGETING plus Non targeting siRNA were purchased by Thermo Scientific (Dharmacon RNAi Technology) and used as specific WASp siRNA and as control siRNA, respectively. BM-pDCs or -cDCs were transfected with 10 µM of siRNA using the Amixa Nucleofector according to the manufacturer’s instructions. We obtained BM-pDCs and -cDCs by MACS cell separation from a total BM-DC culture at day 6. 8 x 10^6 wt BM-pDCs 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 FLT3-L was added to each cuvette after transfection to rapidly dilute the reagents and promote cell survival following shock-electroporation. The transfected pDCs were collected and seeded into 24-well plates containing complete IMDM plus FLT-3L. Cells were collected 48 h after transfection. To assess depletion of WASp 3 x 10^5 cells were lysed and analyzed by SDS page using anti-WASp antibody (Santa Cruz, Biotechnology, Inc.). Protein levels were quantified in Image J.

Cytochalasin D treatment

BM-pDCs and -cDCs were plated in 96-well culture plates in complete medium including 1 µg/ml cytochalasin D (Sigma-Aldrich) for 20 min at 37°C. Cells were washed two times and incubated in complete medium containing the TLR ligands for 5 h at 37°C. Culture supernatants were collected for ELISA analysis.

Functional in vitro assays

Splenic pDCs isolated by sorting or by magnetic beads were washed, resuspended in complete IMDM supplemented with Flt3 ligand, and cultured in 96-well culture plates for different time points at 37°C in the presence of CpG-A or CpG-B at 15 µg/ml. Culture supernatants were collected for ELISA analysis. Cell pellets were lysed to extract RNA for the qRT-PCRs.

Silenced BM-pDCs were washed, resuspended in complete IMDM supplemented with FLT3-L ligand, and cultured in the presence of CpG-A or CpG-B at 15 µg/ml. Silenced BM-cDCs were cultured in the presence of CpG-B at 7 µg/ml. Culture supernatants were collected after different time points for ELISA analysis.
Materials & Methods

For the assays with mice sera, BM-pDCs were resuspended in complete IMDM supplemented with FLT3-L and cultured in 96-well culture plates over night at 37°C in the presence of 1:5 dilution of sera. Culture supernatants were collected for ELISA analysis. Values obtained in control wells (diluted sera without cells) were subtracted from experimental wells to quantify de novo production by pDCs. Treatment with DNase was performed as follows: DNase I (Fermentas) was dissolved in IMDM at 200 μg/ml and 50 μl of serum was incubated with an equal volume of PBS containing 10 μg DNase and 0.3 μmol MgCl₂ for 1 hr at 37°C. Five microliters of 50 mM EDTA was added to stop the enzyme reaction. Control sera were incubated with IMDM containing no DNAse.

For the in vitro maturation assay, BM-cDCs were stimulated with LPS at 0.5 μg/ml at 37°C, and up-regulation of CD86 and CD40 was measured by FACS analysis 4 hrs later.

Antinuclear antibody (ANA) detection

NIH 3T3 cells were grown on glass coverslips in 35-mm dishes. After 1 day in culture at 60% confluence, the cells were washed with PBS and then fixed for 20 minutes at RT with 4% paraformaldehyde in PBS. The fixed cells were permeabilized by exposure to 1% Triton X-100 in PBS for 5 minutes, washed and incubated with blocking buffer (PBS-2%BSA) for 1 hour at RT. Cells were then stained with sera collected from indicated mice (diluted 1:40 in PBS) for 1.5 hours followed by incubation with FITC-conjugated goat anti-mouse IgG antibody for 1 hour (Sigma Aldrich). Cells were subsequently washed, stained with DAPI and the coverslips were mounted on the slides.

Proteinuria evaluation.

Proteinuria was evaluated using Simens multistix 10SG. Proteinuria index was scored as follows: 0 < 30 mg/dl; 1 = 30 mg/dl; 2 = 100 mg/dl; 3 = 300 mg/dl; and 4 ≥ 2,000 mg/dl.

Detection of phosphorylation of Pyk2, paxillin and Src kinases

5x10⁵ of WT BM-pDCs were resuspended in complete IMDM supplemented with FLT3-L ligand and incubated with CpG-A at 10 μg/ml for 10 or 30 min at 37°C. Cells were washed and lysed with NP-40 plus phosphatase inhibitors (20 mM Tris-HCl [pH 7,4], 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM NaF, and Protease Inhibitor Cocktail). Total lysates were analyzed by SDS-PAGE (NuPAGE 4-12% Bis-Tris gradient gels, Invitrogen) and immunoblotted with the antibody Anti-Phospho-tyrosine (P-Tyr-100), and
antibodies specific for phospho-Pyk2 (Tyr-402), phospho-paxillin (Tyr-118) and phospho-Src family (Tyr-416), all provided by Cell Signaling Technology.

Immunoprecipitation of WASp

8x10^6 of WT BM-DCs were resuspended in complete IMDM supplemented with Flt3 ligand, and incubated with CpG-A at 20 μg/ml for 3, 7 or 10 min at 37°C. Cells were washed and lysed with NP-40 plus phosphatase inhibitors. Total lysates were subjected to overnight incubation at 4°C with protein A-Sepharose beads (Amersham/Pharmacia) previously treated for 2hrs with 2 μg of polyclonal anti-WASp (H-250) (Santa Cruz Biotechnology). Beads-antibody-protein complexes were washed four times with lysis buffer and eluted with SDS-PAGE sample buffer. Immunoprecipitated proteins were separated by SDS-PAGE using 7.5% polyacrylamide gel. Antiphosphotyrosine and anti-WASp immunoblotting was performed using an anti-P-tyrosine (P-Tyr-100 (Cell Signaling) and an anti-WASp monoclonal antibody (B-9) (Santa Cruz Biotechnology), respectively.

IFN-α inhibition assay

WT BM-DCs at 6 day of culture were collected, plated in a 96 multiwell plate (5 x 10^5 cells in 100 μl) and incubated with 100 μl of supernatant from wt or WKO BM-DCs cultures in presence or not of a neutralizing IFN-α antibody (200 NU) (PBL Biomedical Laboratories, Inc). After 20 hrs cells were stained for maturation marker CD86 and analyzed by FACS.

In vitro DC:T cells co-culture

WT and WKO BM-DCs (3 x 10^5) were cultured with different forms of ovalbumin (OVA)-associated antigens (OVA expressing-bacteria, OVA-latex beads, or OVA323-339 peptide). After 1 h of pulse, cells were washed, resuspended in medium alone and fixed with PFA 1% after a 4-hours chase period. 1.5 x 10^5 of purified OT-II cells were added to the wells, and after 12 hrs of co-culture the expression of CD69 on OT-II CD4^+ T cells was measured by FACS, and IL-2 present in the supernatant evaluated by ELISA (BioLegend). Preparation of stimuli: *S. typhimurium*- and *E. Coli*-expressing OVA (3.6 x 10^6 CFU) were heat-killed by a treatment of 10 min at 100°C or 70°C, respectively. OVA-latex beads were obtained by conjugating OVA through passive adsorption (4°C over night on rotation) to latex beads (1 micron, from Polysciences, Inc.). All stimuli were resolved in PBS.
For the measure of IL-6 produced by DCs upon co-culture with T cells, WT and WKO BM-Cs (3 x 10^5) were incubated with OVA_{323-339} peptide (1 μg/ml) and mixed with purified OT-II cells for 20 hrs at 37°C. Supernatant was collected and IL-6 measured by ELISA.

T cell differentiation in vivo

Wt and WKO BM-DCs were pulsed with increasing doses of OVA_{323-339} peptide and after 4 hrs washed and plated with purified OT-II. T cell cytokines production (IL-17 and IFN-γ) were measured after 3 days of co-culture by ELISA (ELISA Ready-SET-go! kit for IL-17, BD Pharmingen ELISA kit for IFN-γ).

Immunofluorescence analysis

WT and WKO BM-pDCs or wt BM-pDCs treated with control and WASp specific siRNA were incubated with 3 μM CpG-cy5 (SIGMA) for 15 min. Cells were washed extensively and fixed for immuno-labeling. Alexa-488 phalloidin (Molecular Probes) and goat anti-EEA-1 (Santa Cruz, Inc) followed by anti goat Alexa-555 (Molecular Probes) were used to detect cell area and early endosomes respectively. Analysis of cell area was performed using an automated high-content screening microscope (Molecular Devices ImageXpress Micro). Number of cells (DAPI) and phallodin signal to define cytoplasm area were acquired simultaneously and analyzed. Confocal images were acquired in a LSM510 META Axiovert 200M reverse microscope with a 63x objective. For quantification of endosomes, objects were identified using a cut-off of 0,2 μm^2. 21 zed slices of 0,2 μm were reconstructed using the Volocity software (Perkin Elmer, Inc), and vesicles volumes were measured automatically by the software To quantify CpG-A-cy5 containing vesicles, cells were acquired on channel corresponding to phallidin and then illuminated with the 633 laser to visualize cy5. Only cells containing a cy5 signal above the threshold of intensity and larger than 0,2 μm^2 were analyzed.

Adoptive transfer and T cell activation

For priming with αDEC205OVA, mice were adoptively transferred with 2 x 10^6 of purified OT-I/CD45.1 cells and injected s.c. with 100 ng of αDEC205-OVA plus 100 μg of anti-CD40 in the footpad 24 h later. LNs were collected at day 10 to quantify the percentage of OT-I/CD45.1 by FACS.
Patients and Tissues

The occurrence, frequency and distribution of PDC were studied on formalin-fixed paraffin embedded spleen sections from patients affected by WAS or XLT (11 cases) and obtained from the archive of the Department of Pathology, Spedali Civili di Brescia. Clinical and molecular data were provided for each case (Department of Pediatrics, Institute of Molecular Medicine "A. Nocivelli", University of Brescia, Brescia, Italy) and previously described in (Vermi et al., 1999; Wengler et al., 1995; Jin et al., 2004). Normal spleen from age-matched control patients (10 cases) who underwent splenectomy for trauma or spherocytosis were used for comparison. Peripheral blood samples from patients and age-matched HDs were collected according to the protocol for research studies on biological material from patients with primary immunodeficiency (TIGET 02), including WAS, approved by the HSR Institutional Ethical Committee. Biological specimen from healthy donors (children) were collected and utilized upon informed consent for research purpose in agreement with the rules defined by approved protocols TIGET PERIBLOOD.

Analysis of gene expression profile in PBMCs

mRNA was extracted from PBMCs of WAS patients and healthy donors using RNeasy Mini Kit (Qiagen, CA). Retrotranscription was performed using RT² First Strand Kit (SABiosciences, Frederick, MD). Real-time PCR on cDNA samples was performed following the protocol provided with the kit (Human Interferon α, β Response PCR Array, SABiosciences) on a ViiA 7 Real-Time PCR System (Life Technologies Corporation, CA). The relative expression of target genes was defined by calculating the Δcycle threshold, with respect to the housekeeping gene.

Immunohistochemistry

Four micron formalin-fixed paraffin embedded tissue sections were stained with anti-BDCA2 (Mouse IgG1, Clone 124B3.13, Dilution 1:50, Dendritics, Lyon, France, EU), and anti-MxA (Mouse IgG2a, Dilution 1:500, kindly provided by Dr. O.Haller). Epitope retrieval was obtained by microwave heating in EDTA buffer (pH 8.0), for 3 X 5 minutes, at 750 W for anti-MxA and for 2 X 5 minutes at JET and 3 X 5 minutes at 750 W for anti-CD303. Reactivity was revealed using the EnVision anti-mouse HRP-linked detection kit (Dako, Glostrup, Denmark) for anti-MXA or Bond Polymer Refine Detection (Leica Microsystems Newcastle Ltd UK) for anti-BDCA2 followed by DAB. Distribution of pDC was analyzed by double immunohistochemistry using primary antibody to BDCA2 and CD3 (Rabbit, Clone SP7, Dilution 1:100, Thermo Scientific, Fremont, CA, USA). After completing the first immune reaction against BDCA2, the second immune reaction was
visualized using Mach 4 MR-AP (Biocare Medical, Concord, CA, USA), followed by Ferangi Blue (Biocare Medical) as chromogen. Absolute pDC count was quantified automatically applying a custom-programmed script in Cognition Network Language based on the Definiens Cognition Network Technology® platform (Definiens AG, München Germany). Briefly, digital slides of entire spleen sections immunostained for CD303 were obtained by Aperio ScanScope CS Slide Scanner (Aperio Technologies, Vista, CA, USA) at 40 × magnification and analyzed using Tissue Studio 2.0 (Definiens AG, München Germany). The quantitative scoring algorithm for BDCA2 was customized by using commercially available templates. The algorithm was created to match the requirements of the CD303 staining parameter in human spleen and includes Manual ROI Selection (Draw Polygon). PDC are expressed as number of CD303 positive cells/mm² of spleen area.

Spleen MxA expression was scored as percentage of positive cells: positivity score 1 = <25% of positive cells; score 2 = 25-50% of positive cells; score 3 = > 50% of positive cells; score 4 = >75% of positive cells. As additional strategy we also measured total MxA signal by Tissue Studio 2.0 (Definiens AG, München Germany) on spleen digital slides immunostained for MxA. The algorithm was created to match the requirements of the MxA staining parameter in spleen sections and includes Manual ROI Selection (Draw Polygon). Data are expressed as percentage of spleen area immunostained for MxA.

For figure panel preparation, immunostained sections were photographed using the DP-70 Olympus digital camera mounted on the Olympus BX60 microscope and processed using Analysis Image Processing software (Olympus).

Analysis of pDC frequency in human peripheral blood

Human PBMCs were purified by centrifugation on a density gradient media (Lymphoprep; Axis- Shield, Oslo, Norway). PBMCs were stained with the following anti-human antibodies: APC-conjugated anti-CD3/anti-CD14/anti-CD15/anti-CD16/anti-CD19/anti-CD20/anti-CD56 (lineage cocktail) (BD Biosciences); PE-conjugated anti-BDCA-2 (Miltenyi Biotec) and PE-Cy7-conjugated anti-CD4 (BD Biosciences). The pDC subset was identified as lineage cocktail negative/BDCA-2⁺/CD4⁺ cells gated on live cells. Stained cells were acquired with a FACS CANTO (BD Biosciences) flow cytometer and analyzed with FCS Express software (version 2.3; De Novo Software, Los Angeles, CA). Isotype controls were performed with corresponding mouse Ig.
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, unless otherwise specified.
3 RESULTS
3.1 Analysis of the pDCs compartment in WKO mice

During the last ten years, many efforts using both, mouse models and cells derived from patients, have contributed to substantially increase our understanding of the pathogenesis of Wiskott-Aldrich syndrome (WAS). Still, it remains to be fully elucidated why patients affected by the most severe form of WAS develop in a high proportion autoimmune phenomena. Some manifestations in WAS partially overlap with those of type-I IFN diseases. Based on this, and on the fact that pDCs in WAS have not yet been analyzed, we wanted to investigate whether the pDC/IFN-α axis may play a role in the pathogenesis of WAS autoimmune phenotype.

We started by investigating the presence of pDC/type-I IFN alterations in a mouse model of the disease. Mice that we used were generated in the Alt laboratory by knockout of the WAS protein gene (Snapper et al., 1998). This murine WAS model present high levels of circulating auto-antibodies, develop spontaneous colitis and proliferative glomerulonephritis with immune complex deposition, providing a good model to investigate the cellular mechanisms leading to autoimmune diseases (Snapper et al., 1998; Nikolov et al., 2010; Humblet-Baron et al., 2007; Shimizu et al., 2012).

WASp knockout mice were generated through targeted disruption of the WASP gene by homologous recombination. The WASp gene is X-linked and, therefore, is present in only a single copy in the male TC-1 ES cell line. To disrupt the murine WASp gene, Alt group employed a gene-targeted mutational techniques to insert a neomycin-resistance gene (neo) into exon 7 of WASp in the reverse transcriptional orientation in TC-1 cells. Targeted ES cells were electroporated with the targeting construct and transfectants isolated after positive and negative selection following standard methodology (Mansour et al., 1988). Targeted ES cells were used to inject C57BL/6 blastocysts, and highly chimeric male mice were bred to C57BL/6 or 129Sv female mice to generate F1 offspring. Female WASp<sup>+/−</sup> offspring were bred to 129 wild type male mice to generate germline WASp<sup>−/−</sup> male mice. WASp<sup>−/−</sup> female mice were obtained from mating WASp<sup>−/−</sup> males with WASp<sup>−/−</sup> females and showed no significant differences with WASp<sup>−/−</sup> males. Mice were back-crossed 12 times on a C57BL/6 background. Our presented analyses represent studies of male WASp<sup>−/−</sup> and WASp<sup>+/+</sup> and female WASp<sup>−/−</sup> and WASp<sup>+/+</sup> obtained from the same original breeding (WASp<sup>−/−</sup>♂ x WASp<sup>+/+</sup> ♀). We will simply refer to all WASp-deficient mice as WKO mice.
3.1.1 PDCs frequency, absolute numbers and turnover rate

To investigate the pDCs compartment in WASp null mice, we first analyzed the frequency and the absolute numbers of pDCs in secondary lymphoid organs. Cell suspensions were obtained from spleen and lymph nodes (LNs) of wt and WKO mice and stained for the cytofluorimetric analysis. PDCs were identified on the live CD45\(^+\) cell fraction as cells expressing intermediate levels of CD11c and high levels of B220, PDCA-1 and Siglec-H (Fig. 2 A). We observed that the frequency of PDCA-1/Siglec-H/B220 cells was higher both in the spleen and LNs of mutant mice as compared to wt animals. Moreover, as spleen and LNs were enlarged in WKO mice, the absolute number of pDCs was significantly higher than in wt controls (Fig. 2 B). To understand the origin of increased cell numbers we performed an in vivo BrdU experiment. BrdU is a synthetic nucleoside, analogue of thymidine, commonly used in the detection of proliferating cells in living tissues. BrdU is incorporated into the newly synthesized DNA of replicating cells substituting for thymidine during DNA replication. Fluorophore-labeled antibodies specific for BrdU are then used to detect the incorporated chemical by FACS, thus indicating cells that were actively replicating their DNA. We fed Wt and WKO mice with BrdU in the drinking water. After 7 days mice were sacrificed and the organs collected for the FACS analysis (Fig. 2 C). We found twice as many labeled pDCs in the spleen, lymph nodes and bone marrow of WKO mice, suggesting that WASP is required for normal turnover rates of pDCs cells. We also examined cell death in freshly isolated splenic pDCs. We performed a standard cell death analysis by using 7-Aminoactinomycin D (7-AAD) and Annexin-V staining. 7-AAD is a fluorescent compound that intercalates into double-stranded nucleic acids. It is
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excluded by viable cells but can penetrate cell membranes of dying or dead cells. Annexin V is a calcium-dependent phospholipid binding protein that has a high affinity for the phosphatidylinerine (PS) which flips to the outside of the plasma membrane during apoptosis. Splenocytes from wild type and WKO mice were stained with antibodies to CD11c, B220, PDCA-1 and labeled with FITC-conjugated Annexin-V and 7-AAD. Dead cells were defined as 7-AAD⁺ cells and apoptotic cells as 7-AAD⁺/annexin V⁺. As shown in figure 2 D, the frequency of apoptotic cells among pDCs was higher in WKO mice as compared to control mice. Together these data indicate that pDCs from WKO animals have an enhanced proliferation rate in vivo, and show an increased propensity to undergo spontaneous apoptosis in the absence of any prior stimulation.

Figure 2. Characterization and quantification of pDCs in WKO mice. (A) Representative FACS plots showing pDCs gating strategies. Numbers indicate the percentage of PDCA-1⁺/Siglec-H⁺/B220⁺ cells. (B) Percentage and absolute number of pDCs in spleen and LNs of WT (n=8-10) and WKO (n=8-11) animals are plotted (n=18). The mean ± SEM are indicated. (C) In vivo BrdU labeling. WT and WASp KO mice were fed BrdU in the drinking water for 7 days and then sacrificed. Representative FACS plots showing the proportion of BrdU positive pDCs in spleen, lymph nodes and bone marrow (Left panel). The percentage of BrdU⁺/PDCA-1⁺ cells in the three organs were plotted as mean ± SEM of 4 mice per groups analyzed in two independent experiments. (D) Representative dot plots showing percentage of apoptotic (7AAD⁺/Annexin V⁺) pDCs in spleen of WT and WKO mice in absence of stimulation. *, p<0.05, **, p<0.01, ***, p<0.005.
3.1.2 Basal activation state of pDCs

To further study the steady-state activation profile of pDCs in WKO mice, we examined the expression of MHC class II and co-stimulatory markers CD40 and CD86. Spleen cell suspensions obtained from wt and WKO mice were stained for cytofluorimetric analysis, and pDCs (CD11c<sup>int</sup>/B220<sup>+</sup>/PDCA-1<sup>+</sup>) analyzed for the expression of MHC-II, CD40 or CD86. The FACS analysis showed that WASp-deficient pDCs express significantly higher levels of all markers in spleen, with an increase of around three-fold in the mean fluorescent intensity (MFI) of CD86 and CD40 expression as compared to MFI of control cells. We also measured the basal maturation profile of WKO pDCs in lymph nodes and blood. Among the surface markers previously analyzed, we choose to measure only CD86 as the easiest to detect at basal levels and the most strongly over-expressed in WKO cells. We found that WASp-deficient pDCs expressed higher levels of CD86 also in lymph nodes and blood, further indicating a state of general constitutive activation of pDCs in WKO animals (Fig. 3).

![Figure 3](image)

**Figure 3. Basal expression of maturation markers by pDCs in WKO animals.** Levels of maturation markers CD86, CD40 and MHC-II at steady state were measured by FACS in different organs as indicated. The mean fluorescent intensity (MFI) in individual mice is indicated. Data represent one of four independent experiments with consistent results. *, p<0.05; **, p<0.01; ***, p<0.005.

3.1.3 WKO pDCs constitutively secrete type-I IFN

PDCs are the major class of type-I IFN producing cells that respond to pathogen encounter by rapidly secreting high amounts of this cytokine. Recognition of nucleic acids by TLR7 and
TLR9 occurs in endosomes and leads to production of type-I IFN, proinflammatory cytokines and enhanced expression of co-stimulatory molecules. Therefore, we next examined whether WKO pDCs were functionally active. To this purpose, we analyzed the serum IFN-α content in wt and WKO mice. As shown in Fig. 4 A, without any prior stimulation, we detected circulating IFN-α exclusively in WKO animals. These data suggested that WKO pDCs, besides showing phenotypic maturation, were functionally active in terms of IFN-α production. To confirm this hypothesis, we isolated by cell sorting an equal number of pDCs from spleen and lymph nodes of wt and WKO mice. RNA was extracted from sorted cells and employed for real-time PCR assay using primers specific for ifna4 gene. As shown in Fig. 4 B, we confirmed the presence of IFN-α transcripts only in WKO cells. We tried to measure spontaneous release of IFN-α protein by sorted pDCs in cell culture supernatant. Although protein levels were very low, we observed a consistent trend toward higher IFN-α levels in supernatant of splenic WKO pDCs as compared to wt cells (Fig. 4 C). Thus, we concluded that circulating IFN-α in the sera of WKO animals is not just a consequence of elevated pDCs numbers, but is really due to a constitutive transcription of IFN-α genes by pDCs. As a control we measured the inflammatory cytokine IL-6 (Fig. 4 D). We found that IL-6 sera levels were similar between the two groups of mice.

![Figure 4](image)

**Figure 4. Analysis of the basal IFN-α levels in WKO mice.** (A) The levels of IFN-α were compared in the sera of WT (n=12) and WKO mice (n=19) in absence of any prior stimulation. Values in individual mice and the median ± SEM for each group are plotted. (B) The expression of Ifna4 in pDCs isolated from the spleen and draining lymph nodes of WT and WKO mice at steady state was analyzed by qRT -PCR. CT were obtained by normalizing target gene to the house keeping beta-actin. Values are shown as the $2^{\Delta\text{CT}} \times 10^3$. Data are the means ± SEM of four independent experiments, each with 4 mice per group. (D) Basal levels of IL-6 present in the sera of WT (n=14) and WKO mice (n=13). *, p<0.05, ***, p<0.005.

### 3.2 Analysis of the response to TLR9 stimulation by WASp-null pDCs

Results described in the previous section unveil that pDCs in WASp-deficient animals become spontaneously activated and constitutively secrete detectable amounts of type-I IFN.
To further examine alterations in WKO pDCs in terms of functionality, we next evaluated their ability to respond to TLR9 stimulation.

### 3.2.1 Progressive exhaustion of the TLR9/IFN-α pathway in pDCs from WKO mice

To better understand the functionality of pDCs in WASp-null mice we performed a series of experiments in which synthetic CpG-ODNs were used to trigger TLR9. We first tested pDC maturation in response to challenge in vivo with CpG-B, known to potently induce pDC maturation. Wt and WKO animals were injected intravenously with CpG-B and after 4 hours sacrificed. Spleens were collected and treated for the cytofluorimetric analysis. PDCs from wt animals up-regulated the expression of CD86 upon challenge, reaching levels of expression similar to those shown by pDCs isolated from not injected WKO mice. Notably, no increase in the CD86 expression was detected in WKO pDCs upon challenge (Fig. 5). These data suggested that WKO pDCs undergo spontaneous maturation but are refractory to further TLR9 stimulation.

We next challenged the animals with CpG-A and measured the levels of IFN-α present in sera. Unlike monomeric CpG-B, that induce only moderate amounts of type-I IFN, CpG-A forms multimeric structures capable of leading to a robust and rapid type-I IFN secretion. In order to maximize the IFN-α production, CpG-A was complexed to DOTAP, a cationic liposome that strongly enhances CpG cellular uptake and, therefore, the IFN response. Wt and WKO were challenged intravenously with CpG-A-DOTAP and the sera collected at two different time points.

![Figure 5. In vivo maturation of WASp deficient pDCs. CpG-B was injected in the caudal vein of WT and WKO mice (n=5). After 4h, mice were sacrificed and the spleens collected. Splenocytes were stained for CD11c, B220, and PDCA-1 in combination with CD86. The MFI (gated on the CD11c^{hi}/B220^{-}/PDCA-1^{-} population) for CD86 was determined at FACS. Data represent one of two independent experiments with consistent results. *, p<0.05.](image-url)
In wt mice the levels of IFN-α increased steadily at 2 and 6 hours post injection. In WKO mice, despite higher levels at steady state, the concentration of IFN-α failed to increase upon challenge. More precisely, IFN-α concentrations started to increase in sera at 2 hours post-injection, similarly in both groups, however, at 6 hours post-challenge IFN-α was increased of about 3 folds in wt mice but remained low in WKO mice. This was specific of IFN-α as IL-6 and IL-12p40 levels increased over time and were comparable in the two genotypes (Fig. 6).

To verify that lack of circulating IFN-α in WKO mice was due to unresponsiveness of pDCs we performed two types of experiments: 1) an intracellular staining of pDCs for IFN-α production, and 2) stimulation ex-vivo of pDCs. For detection of intracellular IFN-α, mice were injected intravenously with CpG-A-DOTAP and sacrificed at 45’ or 2 hrs and 30’ post-injection. Cells were enriched from total spleen by depletion of B and T cells, and stained for extracellular markers using antibodies to CD11c, B220 and PDCA-1. Cells were then fixed, permeabilizied and stained over night with a FITC-conjugated anti-IFN-α. Brefeldin-A (BFA), a secretion inhibitor, was added during all the phases of DCs enrichment procedure and staining in order to trap cytokine production and maximize the intracellular IFN-α staining signal. As shown in Fig. 7, IFN-α producing pDCs were detected in the spleen of wt mice as early as 45’ post-injection and increased at 2 hrs and 30’. In contrast, the frequency of IFN-α⁺ pDCs in the spleen of WKO mice was much lower at both time points. Sensitivity of detection by intracellular staining was too low to appreciate basal IFN-α production by WKO pDCs.
In parallel, we performed experiments in which FACS-sorted splenic pDCs were stimulated ex-vivo with CpG-A and –B to quantify type-I IFNs mRNA. As shown in Fig. 8 A (left panel), transcription of IFN-α genes induced by CpG-A was high at 4 hrs in wt cells whereas almost no transcripts were detected in WKO cells. This was not due to a slower kinetic of production as a amounts of IFN-α mRNA were low in WKO pDCs even after overnight CpG stimulation. As expected, CpG-B scarcely induced transcription of IFN-α genes in both genotypes. To confirm that reduced activation of gene expression was reflected in less protein production, sorted-pDCs were put in culture for 12 hrs in presence of CpG-A or-B, and supernatant collected for ELISA analysis. The IFN-α response was abrogated in WKO pDCs at the protein levels as the supernatant of wt pDCs contained significantly higher concentration of IFN-α than WKO cells in response to stimulation with both CpG-A and-B (Fig. 8 B, left panel). To verify whether the inability of WKO pDCs to respond to exogenous TLR9 stimulation was restricted to IFN-α pathway, we tested ex-vivo-stimulated pDCs for the production of IL-6 both at mRNA and protein level. We found that transcription of IL-6 genes increased steadily at 4 hrs and after overnight incubation with CpG-B, and equally in the two genotypes. This result was confirmed at the protein level as the supernatant of wt and WKO pDCs contained similar levels of IL-6 (Fig. 8 A-B, right panels).
To exclude that unresponsiveness of WKO pDCs to CpG was due to reduced expression of TLR9, we tested the levels of TLR9 mRNA in wt and WKO pDCs by real-time PCR assay. PDCs were obtained from total bone marrow-derived DCs (BM-DCs) cultures by using MACS anti-B220 magnetic beads, and the mRNA isolated for the qRT-PCR analysis. As shown in Fig. 9, no differences in the TLR9 transcripts levels were observed between wt and WKO cells.

**Figure 8. Cytokine production by WKO pDCs stimulated ex-vivo with CpG ODNs.** (A) 3 x 10^5 cell sorted splenic pDCs from WT and WKO adult mice were stimulated ex-vivo with CpG-A and CpG-B (15μg/ml) for 4 and 24 hrs (ON). Bars show the relative expression of Ifna4 and Il6 transcripts evaluated by qRT-PCR. The target mRNA was normalized to beta-actin mRNA. Values are shown as the 2^ΔCT x 10^3. Data are the means ± SEM of four mice/group and are representative of three independent experiments. (B) As in (A), cell culture supernatants were collected after 16 hrs to determine IFN-α and IL-6 protein levels by ELISA. Data are expressed as the mean ± SEM of three independent experiments. *, p<0.05, **, p<0.01

**Figure 9. TLR9 levels in WKO pDCs.** Bars show the relative expression of Tlr9 transcripts present in wt and WKO BM-derived pDCs evaluated by qRT-PCR. Data are the means ± SEM of two mice/group and are representative of two independent experiments.
Together these data show that a constitutive activation of the IFN-α pathway in WASp null pDCs leads to progressive exhaustion and inability to respond to further exogenous TLR-9 stimulation.

3.2.2 Analysis of the constitutive pDCs activation in WASp KO mice

Previous results show that pDCs in WASp-null mice are chronically activated but become tolerant in response to further TLR-9 triggering. Chronic pDCs activation with progressive exhaustion of the IFN-α pathway has already been described in SLE patients, where uncontrolled pDCs activation depends on triggering of TLR-9 by nucleic acids-containing immune-complexes (Ronnblom et al., 2003; Barrat et al., 2005). Since high-titer of anti-dsDNA antibodies were reported in WASp-deficient mice (Humblet-Baron et al., 2007) we reasoned that this might represent an endogenous trigger for chronic pDCs activation.

We first analyzed the presence of autoantibodies in sera of WKO animals by an antinuclear antibody (ANA) test. This test was set up to detect IgG anti-dsDNA autoantibodies by performing indirect immunofluorescence on mouse NIH3T3 fibroblasts. Diluted sera from wt, adult WKO (>6 months) and young WKO (<3 weeks) mice were incubated with NIH3T3 cells and the anti-nuclear reactivity was detected using a FITC-conjugated anti-IgG antibody. As shown in Fig. 10 A, we confirmed that sera of adult WKO mice had strong anti-nuclear antibodies that were absent in younger animals. Second, we used these mice sera to stimulate wt pDCs, in order to evaluate their activating ability on pDCs. We found that wt pDCs stimulated with sera of adult WASp-null mice (6-12 weeks), but not with sera of young WKO mice or wt mice, produced IFN-α. (Fig. 10 B).

Moreover, the IFN-α inducing capacity was significantly reduced when sera from WKO adult mice were pretreated with DNAses (Fig.). Together these data suggest that immune complexes of autoantibodies and self nucleic acids constitute an endogenous trigger for chronic activation of pDCs in vivo.
Results

3.2.3 WKO pDCs are intrinsically more responsive to TLR9

So far these results indicate that, similarly to what has been described in SLE, an inflammatory environment capable to activate pDCs establishes with aging in WKO mice. We next analyzed the intrinsic responsiveness of pDCs isolated from young mice (<3 weeks), where there are not endogenous triggers of pDCs. Indeed, we found that WKO pDCs in young mice did not display spontaneous maturation, as maturation markers were expressed at similar levels than in wt counterparts (Fig. 11 A). Surprisingly, when stimulated in vitro with CpG-B, WKO pDCs upregulated CD86 expression more than wt cells (Fig. 11 B).

Figure 10. WKO serum induces IFN-α secretion by pDCs. (A) The presence of IgG anti-dsDNA autoantibodies in sera was analyzed by immunostaining of NIH3T3 cells. Diluted sera from control (WT), WKO (>6 months) and young WKO (<3 weeks) mice were incubated with NIH3T3 cells and the anti-nuclear reactivity was detected using FITC-conjugated anti-IgG antibody (right panels). Nuclei were visualized by DAPI staining left panels. Cells are displayed at a magnification of x40. Representative images from 1 out of 4 sera tested/group are shown. Bars, 36 μM. (B) 3.5 x 10⁵ WT bone marrow derived pDCs (BM-pDCs) were stimulated with sera from the indicated groups of animals treated or not with DNAse I. Secretion of IFN-α was measured by ELISA at 24 hrs. Data are expressed as the values in experimental wells subtracted of the values in control wells (dilutions of sera without cells). Values are means ± SEM of three independent experiments.

Figure 11. Maturation profile of “young WKO” pDCs. (A) Levels of maturation markers CD86 and CD40 at steady state were measured by FACS in spleens of wt and WKO mice younger than 3 weeks. MFI in individual mice is indicated. Data represent one of four independent experiments with consistent results. (B) CpG-B was injected in the caudal vein of WT and WKO mice (n=5). After 4h, mice were sacrificed and the spleens collected. Splenocytes were stained for CD11c, B220, and PDCA-1 in combination with CD86. The MFI (gated on the CD11c⁺/B220⁻/PDCA-1⁺ population) for CD86 was determined at FACS. Ratio between CD86 MFI of mice injected and CD86 MFI of not injected mice is shown.

To better investigate the functionality of pDCs from young animals, we performed experiments in which FACS-sorted splenic “young pDCs” were stimulated ex-vivo with CpG-A and -B to quantify type-I IFNs mRNA. Surprisingly, we found that transcription of IFN-α genes induced by CpG-A
was significantly higher in young WKO pDCs than in pDCs isolated from aged matched wt controls. Increased production was particularly clear after 4 hrs of stimulation, as following overnight incubation only a small amounts of IFN-α transcripts were detectable in both genotypes (Fig. 12 A). To confirm that enhanced activation of gene expression was reflected at protein level, sorted splenic pDCs were put in culture for 12 hrs in presence of CpG-A or-B, and supernatant collected for ELISA analysis. As shown in Fig. 12 B, the IFN-α production was increased in young WKO pDCs even at the protein level as significantly higher levels of IFN-α were measured in the supernatant of WKO cells in response to CpG-A stimulation as compared to control cells. Enhanced response of young WKO pDCs to TLR9 triggering was restricted to IFN-α pathway, since IL-6 production was not affected either at mRNA or protein level. These data suggest that two factors may contribute to constitutive pDCs activation: an intrinsic lower threshold, and the presence of environmental inducers. To test this we stimulated pDCs from young wt and WKO mice with sera derived from adult WKO animals. We found that pDCs isolated from young WKO animals responded more vigorously than pDCs from young wt mice to stimulation with sera derived from adult WKO animals (Fig. 12 C).
Collectively, these data strongly indicate that pDCs in KO mice are hyper-responsive to TLR9 agonists, but become progressively exhausted because of continuous triggering of TLR9 by endogenous pDCs activators.

3.2.4 WASp plays a cell-autonomous role in pDCs

Previous results suggest that WASp KO pDCs are basically hyper-responsive to TLR-9 stimulation and progressively develop a selective exhaustion of the TLR-9/IFN-α pathway because of continuous triggering by endogenous factors. To fully demonstrate that WASp plays a cell-autonomous role in pDCs, in a context that excluded environmental factors, we performed gene silencing assays. In these experiments wt bone marrow-derived pDCs (BM-pDCs) were treated with a specific small interfering RNA (siRNA) targeting mouse WASp (si WASp) or with a negative control siRNA (si irr) with no homology to any known mammalian gene. Transfection of pDCs was performed using a commercial kit and an Amaxa nucleofector machine. Transfection with Amaxa system bases on a new electroporation-based method that enables the DNA to directly enter the nucleus while cells are stabilized in an electric field by treatment with a specific nucleofection solution. We tested in parallel several WASp specific and control siRNAs to individuate those associated to major depletion of WASp expression and minor cell toxicity. As shown in Fig 13 A, the control siRNA 3 and si WASp 9 provided the best siRNAs combination in terms of cell survival and protein depletion (70% of reduction), respectively. We choose this pair of siRNA to perform the next series of experiments. Control and silenced pDCs were stimulated with CpG-A, and IFN-α secretion was measured at multiple time points. We found that upon stimulation the production of IFN-α rapidly increased over time and was significantly higher in WASp-depleted pDCs than in cells treated with the control siRNA (Fig. 13 B, left panel). Interestingly, we observed that a second siWASp that caused a lower reduction in WASp expression (55%) still caused a significant increase in IFN-α production upon 6 hrs of CpG-A stimulation (Fig. 13 C). Depletion of WASp caused activation selectively of IFN-α pathway, as, in contrast, the levels of IL-6 following CpG-B stimulation were only slightly increased (Fig. 13 B, right panel).
To finally demonstrate that WASp plays a cell-autonomous role specifically in pDCs subset, we performed a silencing experiment in conventional BM-DCs (BM-cDCs). Control and silenced cDCs were stimulated with CpG-B, and the inflammatory cytokine content was measured at multiple time points. As shown in Fig 14 B, depletion of WASp in cDCs did not associate to differences in the levels of IL-6 and IL-12 p40 following CpG-B stimulation.
Thus, altogether these results indicate that WASp expression is required to control and restrain activation of the IFN-α pathway in pDCs, and that a depletion of WASp, even if partial, is capable to lower the threshold and lead hyper-responsiveness.

3.2.4 WASp-mediated actin dynamics selectively control TLR9/IFN-α axis in pDCs

From the above results, it has emerged a cell-autonomous role of WASp in controlling the IFN-α pathway activation. Since WASp is a critical regulator of actin polymerization events in hematopoietic cells, we moved to investigate whether WASp may restrain excessive IFN-α activation via its actin regulatory properties. We treated wt BM-pDCs with cytochalasin-D, a fungal toxin which binds to the barbed ends of actin filaments strongly inhibiting actin polymerization. Similarly to what happens in the absence of WASp, we found that cytochalasin-D-treated pDCs produced a large increment of IFN-α levels upon CpG-A treatment (Fig. 15 A). This was selective for IFN-α since IL-6 secretion was not affected (Fig. 15 B). Also the release of proinflammatory cytokines by conventional DCs was not modified by cytochalasin-D treatment (Fig. 15 C).
Results

We found that interfering with actin polymerization via a different way induces an increase of IFN-α in pDCs. Therefore, we conclude that WASp has a role in restraining excessive activation of pDCs/IFN-α axis via its actin regulatory properties.

3.3 Analysis of the endocytic pathway in WASp-null pDCs

Data shown in the previous section revealed that, functionally, WASp-deficient pDCs are hyper-responsive to TLR9 stimulation when isolated from mice younger than 3 weeks, but become progressively unresponsive with mice aging. We found that autoantibodies that accumulate over time in sera of WKO mice act as an endogenous triggers of pDCs activation explaining, at least in part, the ongoing type-I IFN production and the refractoriness to secondary challenges. Beside the contribute of cell-extrinsic environmental factors to chronic WKO pDCs activation, we unveiled an intrinsic WASp-dependent regulation of TLR9 signaling pathway. We found that interfering with actin polymerization or silencing WASp lead to selective amplification of IFN-α production in pDCs, despite normal production of inflammatory cytokines and cDCs activation. So, we concluded that WASp and actin limit selectively IFN-α production in pDCs.
The unique ability of pDCs to produce large amounts of type-I IFN depends on recognition of TLR9 ligands in endosomes and on spatio-temporal segregation of signaling components within the endocytic pathway. The prolonged retention of large DNA aggregates in early endosome provides a platform for extensive activation of the signalling complex, including MyD88 and IRF-7, which leads to a robust and rapid type-I IFN production (Honda et al., 2005; Guiducci et al., 2006). At the same time, recent reports showed that many steps of the endocytic process such as invagination of the plasma membrane, vesicle detachment and splitting, fission and maturation of endosomes into lysosomes are regulated by actin nucleation-promoting factors members of the WASp family (Ohashi et al., 2011; Derivery et al., 2009). Therefore, we speculated that WASp might regulate type-I IFN production by controlling the correct architecture of the endocytic pathway.

3.3.1 WASp-mediated actin dynamics regulate accumulation of CpG-A in early endosomes

To address whether WASp might control endosomal actin, thereby affecting endosomal retention and signal strength, we firstly examined the role of WASp during pDCs activation. We performed a series of experiments to establish how WASp mediates signaling downstream TLR9 activation in pDCs. Firstly, we stimulated cells with CpG-A to study modifications of cytoskeleton proteins related to WASp that were shown to be regulated by TLR signaling in other cell types such as macrophages and myeloid DCs (Sanjuan et al., 2006). Wt BM-pDCs were stimulated with CpG-A for 10 or 30 min, and cell lysates were then analyzed by immunoblotting with an anti-phosphotyrosine (pTyr) antibody. As shown in Fig. 16 A, CpG-A treatment induced rapid tyrosine phosphorylation of multiple proteins. Among the major phosphoproteins individuated, the Src family kinases (SFKs) (60 kDa), the hematopoietic-specific focal adhesion kinase Pyk2 (125 kDa), and paxillin (68 kDa) were further specifically identified by immunoblot with corresponding antibodies (Fig. 16 B). These proteins are known to be required for cellular responses that involve rearrangement of the actin cytoskeleton. For example, the phosphorylation of Pyk2 by SFKs promotes actin rearrangements that induce cell adhesion, spreading, and lamellipodia formation in macrophages (Suen et al., 1999). LPS-stimulated phosphorylation of paxillin by activated SFKs controls spreading and adhesion of human monocytes (Williams and Ridley, 2000). Therefore, the phosphorylation of SFKs, Pyk2 and paxillin upon CpG stimulation suggested that TLR9 signalling plays an important role in actin reorganization in pDCs. To further investigate a direct role of
WASp in actin rearrangements during TLR9 activation we analyzed the phosphorylation of WASp upon CPG-A treatment. WASp phosphorylation is a mechanism that was shown to be essential for WASp functions in several model systems (Cory et al., 2003). Phosphorylation of the tyrosine residue 291 has been reported to be necessary for WASp effector functions downstream of the T cell receptor including efficient actin polymerization, immunological synapse (IS) formation, and T cell activation, as well as for phagocytic cup formation in macrophages and generation of osteoclast sealing zones (Badour et al., 2004; Tsuboi et al., 2007b; Chellaiah et al., 2007; Locci et al., 2009). WASp phosphorylation has been reported to be associated to TLR4 stimulation (Sakuma et al., 2012), but no data have emerged yet regarding a link to TLR9 signaling. We immunoprecipitated WASp from BM-DCs stimulated for different time points with CpG-A, and then the profile of phosphorylation was revealed by incubation with an anti-pTyr antibody. As shown in Fig. 16 C, we found that CpG-A induces rapidly phosphorylation of WASp, with maximum phosphorylation achieved 7 min after stimulation. This result demonstrate for the first time that the protein is implicated in signaling downstream of CpG-A.

Figure 16. CpG-A induces tyrosine phosphorylation of actin regulatory protein and WASp. (A) and (B) WT BM-pDC were stimulated with CpG-A for the indicated periods of time. The whole cell lysates of pDCs were then Western blotted with an anti-phospho-Tyr, anti-phospho paxillin, anti-phospho Pyk2, and anti-phospho Src antibodies. (C) WT BM-pDC were lysed after the indicated times of stimulation with CpG-A. WASp was immunoprecipitated from the cell lysates and the anti-WASp immunoprecipitates were Western blotted with anti total phospho-tyrosine and anti-WASp antibodies.
We next performed an immunofluorescence analysis to pinpoint the localization of WASp in CpG-A activated pDCs (Fig 17). We observed a shifting of the WASp labeling from a diffuse cytoplasmic distribution in resting pDCs to intense accumulation of WASp in early endosomes upon activation. This suggested that WASp is involved in remodeling of endosomal actin during pDCs activation by TLR9 agonists.

![Figure 17. CpG induces localization of WASp in early endosomes.](image)

3.3.2 WASp regulates the correct organization of the endocytic pathway

Previous results showed that WASp is involved in remodeling of endosomal actin during TLR9 stimulation of pDCs. Therefore, as next step, we moved to analyze endosome structure and CpG localization in wt and WKO cells. We labeled early endosomes in not stimulated BM-pDCs. We found that resting wt pDCs contained several well-defined rounded vescicles with an average volume of 2.9 μm³ (±3.21 SD) that were distributed uniformly within the cell. In contrast, WKO cells often contain a single large vescicle (5.3 μm³ ±4.8 SD) at one pole of the cell, suggestive of clustering of several fused endosomes. We acquired z-sections of individual cells to quantify the volumes of EEA-1⁺ vescicles on 3D reconstructed images, and we found a significant increase in the size of EE in WKO pDCs as compared to control cells (Fig. 18).

![Figure 18. Enlarged endosomes in WKO pDCs.](image)
When we stimulated pDCs with CpG-A we saw that rapidly upon triggering (15 minutes) the differences in cell shape and organization of the endocytic pathway become more remarkable. Wt cells lost the regularly rounded morphology, emitted few cell protrusions and showed only a moderate increase in cell area. In contrast, many WKO pDCs adopted a very flatten and extended morphology with large lamellipodia that led to an important increase in total cell area (Fig. 19).

Next we tracked the fate of CpG-A labeled with Cy5. We fed the cells for 15 min and then we performed the immunofluorescence analysis using phalloidin and EEA-1 staining. We found that wt pDCs showed the expected overlap between CpG-A and early endosomes and a patch of actin in the region of uptake. On the contrary, in WKO cells the overall cellular architecture was deeply modified. We observed a global change of the endocytic compartment with large clusters of endosomes in the perinuclear area and the appearance of tubular endosomes in WKO cells. Strikingly, we found very large aggregates of CpG-A within the cytosol of WKO pDCs contained within vesicles surrounded by a weak EEA-1 staining. By quantifying the volumes of CpG-A containing vesicles on 3D reconstructed images we found that intracellular CpG-A clusters had a significantly larger size in WKO than in WT (Fig. 20 A-B).

Figure 19. Increased cell area and large lamellipodia in WKO pDCs. WT or WKO pDCs were treated with CpG-A for 15 min and labeled with phalloidin. (A) Images show a field (40x magnification) and a zoom on a single cell, representative of WT and WKO pDCs. (B) Bars show the mean cell area (± SD) calculated automatically on 1543 WT and 1266 WKO pDCs cells using a high-content screening microscope. ***, <0.0001, Mann-Whitney test.
To further demonstrate that alteration of the endocytic pathway depends on WASp expression, we studied the CpG-A localization and the EE structure in WASp-silenced pDCs. The overall morphology of siRNA-treated pDCs was different, with cells displaying higher numbers of smaller endocytic vesicles with respect to not transfected cells, regardless of the siRNA specificity. Upon exposure CpG-A-cy5 cells were labeled and analyzed by confocal. Similarly to what we had found in WKO cells we noticed the presence of several cells bearing very large aggregates of CpG-cy5 in pDCs treated with WASp siRNA (Fig. 21 A). We acquired z stacks of several control and siWASp cells to quantify the volumes of CpG-A positive vesicles and, consistently, we found a statistically significant increase in WASp silenced cells (Fig. 21 B).
We conclude that lack of WASp in pDCs causes endosomes enlargement in resting cells and the accumulation of large aggregates of TLR9 ligands upon stimulation. Therefore, we propose that abnormal endocytic trafficking in WASp deficient pDCs may account for sustained signaling and increased activation of type-I IFN genes.

**3.4 PDCs/type-I IFN alterations in WAS patients**

Results described in the previous sections show that a sum of extrinsic and intrinsic components makes pDCs in WASp-null mice chronically activated and capable to constitutively secrete type-I IFN. In fact, on one hand, we found the presence of cell-extrinsic factors in sera of WASp null adult mice capable to trigger pDCs activation, similarly to what has been described in SLE. On the other, we unveiled that lack of WASp amplifies IFN-α production downstream of TLR9 in a cell-intrinsic fashion through regulation of the endocytic pathway architecture.

Spontaneous and unabated pDCs activation in WASp-null animals results in ongoing transcription of IFN-α genes. This is of great relevance as increased levels of type-I IFN may be a
crucial initiating factor for the development of WAS autoimmunity, the less understood aspect of the disease. Although type-I IFN secretion is critical for an efficient antiviral response, recent studies indicate, in fact, that high levels of type I-IFN in the absence of infection may result in the establishment of a chronic inflammatory environment that predisposes to autoimmune manifestations. This is widely documented in several human diseases, like SLE, Sjøgren syndrome and psoriasis, where type-I IFN has been shown to promote abnormal/autoimmune responses by a multitude of effects on both the innate and adaptive immune systems.

To demonstrate a link between the alterations in the pDC/IFN-α axis and the pathogenesis of WAS autoimmunity we firstly analyzed the presence in WAS patients of clinical aspects and features known to be associated with type-I IFN-driven autoimmune diseases. Despite the low incidence of the disease (4.1 cases per 1 million live births) we were able to recruit an appropriate number of patients to produce statistically reliable results. We could, in fact, count on a net of collaborators that provided a large number of archival tissues (Dr. W. Vermi, Brescia) and blood samples (Dr. A. Villa, Milano) from WAS patients.

3.4.1 PDCs in spleen and peripheral blood of WAS patients

The most typical manifestations of autoimmune diseases like SLE and Sjøgren syndrome include skin lesions, vasculitis, renal diseases, arthritis and circulating auto-antibodies. All these clinical features are caused by a chronic inflammatory environment initiated and exacerbated by unrestrained levels of type-I IFNs. Since these symptoms overlap with those developed by a high proportion of patients with severe forms of WAS, and given the alterations in the pDC/IFN-α axis found in the mouse model of the disease, we wanted to verify whether human WAS patients present signs of dysregulated pDCs function. We first analyzed spleen sections and peripheral blood of WAS patients. Vermi et al previously reported a general depletion of the splenic white pulp in WAS spleen. However this analysis was limited to lymphoid cell populations with no data on the occurrence and distribution of pDCs (Vermi et al., 1999). The availability of an anti-CD303 capable to specifically identify human pDC on fixed tissues allowed to analyze this cellular population of archival samples. This analysis showed that pDCs are detectable in spleens of WAS patients and their localization is as expected in the T cell area, as visualized by double immunostaining for CD3, marker of T cells, and CD303 (Fig. 22 D). The number of pDCs was highly variable in both sets of samples. Of note, we found two WAS samples in which pDCs were absent. Given the heterogeneity, the differences in numbers did not reach statistical significance.

We next analyzed pDC frequency in blood. The analysis of pDC frequency in the peripheral blood
of 11 pediatric WAS patients with a severe clinical score (3-5) revealed a reduction in the frequency as compared to healthy donors (Figure 22 H). These data resemble what observed in SLE, where patients have decreased frequencies of circulating pDCs (Migita et al., 2005) but increased numbers of pDCs in injured peripheral tissues (Blomberg et al., 2001).

Figure 22. Characterization of plasmacytoid dendritic cells in spleen and peripheral blood of WAS patients. Spleen sections are obtained from WAS patients (A-E) and CTR (F) and stained for CD303 (A-D, brown), CD3 (D, blue) and MxA (E and F). Similarly to control cases, the spleen content of CD303− cells in WAS patients is extremely variable (WAS#2 in A, WAS#1 in B; G). CD303− show a typical PDC morphology (WAS#2 in C) and are regularly distributed mainly in T-cell area (WAS#2 in D). E and F illustrate two cases of MxA score 4 (WAS#1) and score 2 (CTR#2). Original Magnification 100x (A, B, D, E and F, scale bar 200 micron) and 600x (C, scale bar 33 micron). (H) The percentage of pDCs in PBMC of 12 WAS patients and 6 age-matched healthy donors was calculated dividing the number of lineage negative/BDCA-2−/CD4+ cells by the number of total live cells. Horizontal bars indicate mean value. *, p<0.05.
3.4.2 Type-I IFN signature in WAS patients

A type-I gene expression IFN signature has been reported in several autoimmune disorders such as SLE (Han et al., 2003), arthritis (van der Pouw Kraan et al., 2007), dermatomyositis (Baechler et al., 2007), systemic sclerosis (Tan et al., 2006) and Sjögren's syndrome (Gottenberg et al., 2006). Therefore, to directly assess a possible role of elevated type-I IFN in WAS, we analyzed the expression of type-I interferon inducible genes in WAS patients by two complementary approaches. In the first, as surrogate marker of type-I interferon production we directly assessed the protein level of MxA on human spleen by immunohistochemistry as described in Mat&Meth. Interestingly, we detected an elevated expression of MxA (score≥3) in 5 out of 8 WAS also confirmed by automatic morphometric analysis (Fig. 22 E-F). Significantly, in 3 of these cases, elevated MxA expression correlates to the occurrence of autoimmunity (Table 1). To extend and strengthen this observation, we extracted RNA from blood samples obtained through our collaborators in Milano. in a cohort of seven WAS patients with a clinical score 4-5, we analyzed the expression of type-I interferon genes and of type-I IFN-inducible genes chosen among those found over-expressed in SLE patients (Bennet et al., 2003). Notably, we observed increased expression of IFN-α genes in six of seven patients analyzed (Fig. 23 A). Moreover, the mean expression levels of five type I IFN-induced genes were significantly higher in this cohort of patients compared with age matched healthy donors (Fig. 23 B). We conclude that WAS patients exhibit a “minimal IFN-I signature” and display increased levels of systemic IFN-α. Overall these data strongly suggest that alterations in the pDC/IFN-α axis can contribute to pathogenesis of autoimmune manifestations in WAS patients.

Figure 23. Gene expression profiling in WAS PBMC. (A-B) mRNA was extracted from PBMCs of WAS patients and healthy donors. Data show expression of three IFN-α transcripts (A) and five IFN-1 inducible genes (B). Gene expression was evaluated by real-time PCR. ΔCt values were calculated for each target gene by normalizing the Ct value of the target gene to the Ct value of the housekeeping gene. ΔΔCt values were calculated for each target gene by normalizing the WAS patient ΔCt value to the mean ΔCt value of the same gene expressed by healthy controls. Fold increase values were calculated for each target gene using the following formula: 2^ΔΔCt. Horizontal bars indicate the mean. Horizontal dashed line indicates healthy donor value.
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Table I. PDC numbers and MxA expression in WAS spleen ne= not evaluable
3.5 Evidences of a link between dysregulated PDCs/type-I IFN axis and altered innate and adaptive immunity in WKO mice

Unrestrained levels of pDCs-derived type-I IFN represent a crucial step in the pathogenesis of the autoimmune manifestations in diseases like SLE, Sjorgen’s syndrome and psoriasis. Our data in WAS patients and WKO mice strongly support the involvement of IFN-α in WAS pathogenesis. In this last section, we will present a set of various analysis in WKO mice that address key innate and adaptive immune pathways known to be targets of elevated type-I IFNs, and whose abnormal function is implicated in the autoimmunity establishment and exacerbation.

WKO mice represent a good model to investigate the causes of WAS-associated autoimmunity. Mutant mice present high levels of circulating auto-antibodies, develop spontaneous colitis and proliferative glomerulonephritis with immune complex deposition (Snapper et al., 1998; Nikolov et al., 2010; Humblet-Baron et al., 2007) In addition, in line with what observed in mice model of SLE and Sjogren syndrome, we found that WKO mice exhibit proteinuria and a significant infiltration of B, T cells and DCs in salivary glands. Proteinuria means the presence of an excess of serum proteins in the urine, and is one of the best indicator of kidney disease or renal involvement in autoimmune disease (Clynes et al., 1998; Theofilopoulos and Dixon, 1985). For example, the NZB/NZW mice, the most studied animal model of human SLE, have high levels of circulating autoantibodies and immune complexes, which interact with Fcγ receptors in the kidney to induce nephritis with consequent proteinuria. By using standard urine test-strip, we found a moderate proteinuria index in WKO animals indicative of early stages of renal damage (Fig. 24 A). For the evaluation of infiltrated cells, total cell suspension were obtained from pulled salivary glands after tissue digestion and then labeled for FACS analysis. As shown in Fig. 24 B, WKO animals presented a statistically significant increase in the percentage of B (B220⁺), T (CD3⁺) cells, and DCs (CD11chi), a finding indicative of a general hyper-activated cell phenotype (Fig. 24 B). Finally, WKO animals often showed marked splenomegaly, another clinical sign frequently present in murine model of autoimmunity (Ochiai et al., 2000) (Fig. 24 C).
Elegant studies with spontaneous mice models of lupus demonstrated that pleiotropic IFNα/β functions are mediated by IFNAR signaling. Generating NZB or C57BL/6-Fas<sup>lpr</sup> SLE mice lacking the α-chain of IFNα/βR, the common receptor for type-I IFN, led to significant amelioration of autoimmune manifestations, including reduced hemolytic anemia, anti-DNA autoantibodies, kidney disease and splenomegaly. Lupus-ameliorating effects were in parallel accompanied by decrease of B and T cell proliferation and reduced DCs maturation and T cell stimulatory activity, conclusively demonstrating a role of type I IFN as mediator in the autoimmune pathogenesis through immunocytes stimulating property. These findings, along with our previous evidences, prompted us to explore in WKO mice phenotypical and functional aspects of immune cell subsets on which type I IFN exerts activating effects.

3.5.1 Conventional DCs (cDCs) phenotype in WKO mice

IFN-α/β promote normal and abnormal (autoimmune) responses by a multitude of effects on both the innate and adaptive immune systems. A major effect is on cDCs in terms of enhanced activation and improved antigen presentation activity. So, we started by testing the maturation of cDCs in vivo. Wt and WKO animals were injected intravenously with soluble LPS, an agonist of TLR4, or with the bacterial model antigen Salmonella typhymurium. After 4 hours mice were sacrificed and the spleen collected for the FACS analysis. We found that in WASp-null mice cDCs, identified as CD11c<sup>hi</sup> cells, expressed significantly higher levels of CD86 and CD40 at steady state,
and that injection of both LPS and intact bacteria induce a further statistically significant increase in the levels of both maturation markers (Fig. 25).

Figure 25. In vivo maturation of WASp deficient cDCs. *Salmonella typhimurium* or LPS were injected in the caudal vein of WT and WKO mice. After 4 hrs, mice were sacrificed and the spleens collected. Splenocytes were stained for CD11c in combination with CD86 and CD40. The MFI gated on the CD11c^{hi} population for the indicated maturation markers were determined. Each symbol represent an individual mouse, means ± SEM are indicated. *, p<0.05, **, p<0.01.

This maturation profile was mirrored in vitro by cDCs differentiated from bone marrow (BM), both at basal level and upon TLR4 stimulation by LPS (Fig.26).

Figure 26. Maturation profile of WKO cDC in vitro. Wt or WKO DCs were stimulated for 4 hours with LPS and then the levels of CD86 and CD40 by cDCs were determined by FACS gating on CD11c^{hi} cells. Bars represent MFI ± SEM from 3 independent DCs cultures, each with 2 mice/genotype.

This may depend on the presence of IFN-α in the culture of WKO BM-DCs. In fact, to generate these BM-DCs, BM precursors were cultured with FMS-like tyrosine kinase 3 ligand (FLT3-L), a cytokine that supports the generation of both, pDCs and cDC phenotypically and functionally equivalent to those identified in vivo in mouse spleen (Naik et al., 2005). To test this we neutralized IFN-α by adding a neutralizing IFN-α antibody in the WKO SN used to stimulate cells. As shown in Fig. 27, WKO SN neutralized for IFN-α was less efficient in inducing up-regulation of CD86 on cDCs.
3.5.2 cDCs functionality in WKO mice

We next assessed cytokine production by wt and WKO cDCs in vivo. Wt and WKO animals were injected with LPS to trigger TLR4 responses. Sera were collected 4 hours later and the levels of IL-12p40 and IL-6 were determined by ELISA. We found that the levels of all three inflammatory cytokines resulted significantly higher in mutant mice upon challenge, whereas cytokine levels at steady state were comparable in the two genotypes. To understand whether the increased cytokine production in KO mice were intrinsically due to enhanced secretion by cDCs we next assessed cytokine production by sorted bone marrow-derived cDC (BM-cDCs). Wt and WKO cDCs were exposed to LPS for a 24 hours period, then IL-12p40 and IL-6 present in the cell culture supernatant were quantified by ELISA. Results showed in KO cells a slight increase in the steady state level of cytokines, and a further statistically significant increase in the cytokine production upon LPS stimulation in WASp-null cells (Fig. 28).

Figure 27. IFN-α contained in WKO cultures induce maturation of wt cDCs. WT BM-cDCs (6 day of culture) are stimulated overnight with supernatant derived from WKO BM-DCs culture, in presence or not of a neutralizing IFN-α antibody. Bars show MFI of CD86 gated on cDCs (B220<sup>-</sup>/CD11c<sup>+</sup>) population. Values are means ± SEM of experimental duplicates, and are representative of two experiments. *, p<0,05.

Figure 28. Cytokine production in WKO cDC. (A) Wt and WKO mice were injected with 100 ng of LPS in the tail vein. Sera were collected 4 hr after and the levels of IL-12p40 and IL-6 were determined by ELISA. Dots represent individual mice, means ± SEM are indicated. (B) Wt or WKO BM-cDC were incubated with soluble LPS. Cell culture supernatant was harvested 24 hr later and the levels of IL-12p40 and IL-6 measured by ELISA. Bars represent mean of three experimental points. Shown is one representative experiment of at least three. *, p<0,05, **, p<0,01.
This results suggest that cDCs conditioned by an inflammatory environment possibly due to IFN-α respond with higher levels of cytokines to further stimulation.

3.5.3 B cells phenotype in WKO mice

Type-I IFN also exert several direct activating effects on B cells relevant to the pathogenesis of systemic autoimmunity, including B cell activation, Ig isotype switching, as well as expansion of B1 cells, thought to be major producers of autoantibodies. Direct effects of type-I IFN add up to indirect effects mediated by IFN-activated cDCs and monocytes, which promote B cell survival and differentiation through secretion of specific activating cytokines. We examined the steady state profile of B cells from WKO mice. We measured the maturation of splenic wt and WKO B cells by analyzing expression of CD69 and MHC-II at FACS. As shown in figure 29, we found indication of a more activated basal phenotype in B cells from WKO as compared to wt cells.

3.5.4 CD4+ T cell responses in WKO mice

To determine whether enhanced activation of WASp-null cDC was associated to increased antigen presentation activity we performed a series of T cell activation assays using ovalbumin (OVA) as antigen model. As reporter cells we used OT-II T lymphocytes, CD4+ T cells transgenic for an αβ TCR that is specific for the OVA323-339 peptide determinant presented in the context of MHC-II I-A^b (Barnden et al., 1998). We examined WKO cDCs for their ability to activate OT-II cells using three different forms of OVA. These forms included OVA expressed in bacteria, OVA bound to latex beads, and the OVA323-339 peptide. T cell activation was measured using two approaches, up-regulation of the activation marker CD69, and production of IL-2 by T-cells.

BM-DCs at 8 day of culture, when the cDCs frequency is around 75% of total cells, were pulsed 1 hour with the OVA-associated antigen, washed and resuspended in medium alone. Cells were fixed after 4 hours chase period and OT-II CD4+ T cells were added to the wells. After 12 hours of co-

*Figure 29. Maturation profile of WKO B cells. Levels of CD69 and MHC-II expressed by B cells (CD11c+/B220+ cells) at steady state were measured by FACS in spleens of wt and WKO mice. MFI in individual mice is indicated. Data represent one of four independent experiments with consistent results *, p<0.05.
Results

culture expression of CD69 on T cells was measured by FACS and the IL-2 production analyzed in parallel by ELISA. As shown in figure 30 A, WASp-null cDCs incubated with both OVA-expressing bacterial models, *S. typhimurium* and *E. Coli*, induced, despite reduced internalization (Catucci et al., 2011), higher levels of CD69 on OT-II cells than wt cells. Similarly, IL-2 produced by T cells 24 hours after incubation revealed higher activation when T cells are stimulated by antigen-pulsed WASp-null cDCs. This higher ability of WKO cells to activate OT-II cells was not limited to antigens that contain ligands for TLRs, because also when incubated with OVA-latex beads WASp-null cDCs induced higher T cell activation (Fig. 30 B). Finally, enhanced antigen presentation was not due to improved antigen processing ability of WKO cells, since the incubation with synthetic OVA323–339 peptide, that bypasses the intracellular antigen processing step, resulted again in increased T cell activation (Fig. 30 C).

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**Figure 30. Antigen presentation by WASp-deficient DCs.** MHC class-II-restricted antigen presentation of OVA-associated antigens. (A) Wt and WKO BM-DCs were pulsed with 3,6 x 10^6 CFU of heat-killed *S. typhimurium* (OVA-S. T.) or *E. Coli* (OVA-E. Coli) expressing OVA. After 1 hour of pulse cells were washed, resuspended in medium alone and fixed after a 4-hours chase period. OT-II CD4^+^ T cells were added to the wells, and after 12 hours of co-culture expression of CD69 on CD4^+^ T cells was measured by FACS and IL-2 present in the supernatant was analyzed by ELISA. Representative dot plots are shown to indicate the percentages of activated CD4^+^ T cells (CD4^+^/CD69^+^) (left panel). Bars represent average values of three experimental points ± SEM, shown is one representative experiment of at least three. In parallel, BM-DC were stimulated with increasing doses of OVA-conjugated latex beads (B) or with OVA323–339 peptide (C). Graphs show mean values of two experimental points ± SEM. Values are representative of three independent experiments with similar results. *, p<0.05, **, p<0.01.
In sum, these data unveil that cDCs from mutant mice show spontaneous activation and a further enhanced maturation response following TLR-4 stimulation. This is reflected in superior ability to activate antigen specific CD4+ T cells.

These findings were highly relevant in light of the prominent role that activated DCs play in promoting differentiation of CD4+ T cells toward Th17, a subset of helper cells linked to several human autoimmune and inflammatory diseases (Bettelli et al., 2006; Cua et al., 2003; Langrish et al., 2005; Nakae et al., 2003). Recent studies show in fact that ligation of CD40 in activated DCs by antigen-specific CD4+ T cells amplifies the DC production of IL-6 that in turn induces Th17 polarization (Iezzi et al., 2009). Therefore, we reasoned that more activated WKO DC may represent a main source of cytokines to promote differentiation of CD4+ T cells toward the Th17 cell subset. To test this hypothesis, we first measured IL-6 secretion in wt and WASp-deficient DCs upon antigen-specific cross talk with CD4+ T cells. Wt and WKO DCs were stimulated with OVA323-339 peptide and mixed to OT-II cells. The IL-6 content in the cell culture supernatant was measured after 24 hours of interaction. As shown in Figure 31, we detected a much higher IL-6 production upon addition of OT-II cells to KO DCs.

Once assessed that WASp KO DCs up-regulate IL-6 production when interacting with antigen specific CD4+ T cells, we moved to analyze the consequences of this altered cytokine milieu in directing CD4+ T cell fate. We performed a preliminary experiment in which wt and WASp KO DCs were stimulated with increasing doses of OVA323-339 peptide and mixed to OT-II cells. After 72 hours of co-culture the T cell cytokines IL-17 and IFN-γ present in the supernatant were evaluated by ELISA. As shown in Figure 32, release of IL-17 was significantly higher when
OT-II were primed by WKO DCs. This was specific of IL-17 as IFN-γ production was comparable in both co-cultures.

![Graphs showing IL-17 and IFN-γ production](image)

**Figure 32. Th17 polarization induced by WKO DC.** WT and WKO BM-DC are stimulated with increasing doses of OVA\textsubscript{323-339} peptide for 4hrs, then washed and mixed to OT-II cells. After 16 hrs supernatant of co-cultures was collected and IL-17 (A) and IFN-γ (B) measured by ELISA. Mean ± SEM of experimental triplicates are shown. Graphs show one of three experiments with consistent results. *, p<0.05, **, p<0.01.

All together these data led us to propose that high levels of type-I IFN present in KO mice chronically exert activating effects on cDCs with a resulting aberrant profile in terms of phenotype and functionality. Hyper-activated cDC promote in turn exaggerated CD4\textsuperscript{+} T cell activation and promote polarization toward the Th17 subset.

### 3.5.5 CD8\textsuperscript{+} T cell responses in WKO mice

As IFN-α/β are known to exert potent effects on CD8\textsuperscript{+} T cells in terms of enhanced survival, and improved activation and proliferation rate, we moved further to monitor CD8\textsuperscript{+} T cell responses generated in WKO mice. We used a previously described system to deliver Ag to DCs in vivo. In
this method the mice are injected with OVA incorporated into a monoclonal antibody to DEC205 (αDEC205-OVA), a C-type lectin family receptor highly expressed by DCs within the T cell area of lymphoid tissues. Immunization with αDEC205-OVA leads to cross-presentation of the MHC class I epitope of OVA mainly by CD8\(^+\) resident DCs and require the co-administration of anti-CD40 mAb to induce long term CD8\(^+\) T cell responses. Targeting immature, steady state DCs via DEC-205 results in fact in the induction of peripheral tolerance rather than immunity (Hugues et al., 2004; Bonifaz et al., 2002). To exclude interference by defective activation of T cells, we analyzed responses of OT-I cells, that are of wt origin and carry a transgenic TCR specific for the OVA SIINFEKL peptide restricted by H-2K\(^b\) (Hogquist et al., 1994). To track the T cells in vivo, we isolated OT-I CD8\(^+\) cells from OT-I mice expressing a specific isoform of the CD45 marker (CD45.1) and adoptively transferred into wt and WKO mice expressing the CD45.2 isoform. We next evaluated the number of OVA-specific T cells by means of an antibody that specifically recognize the CD45.1 isoform.

Wt and WKO mice were adoptively transferred with 2 x 10\(^6\) OT-I cells and s.c. immunized with αDEC205-OVA, plus the adjuvant anti-CD40 mAb. As control, mice were injected only with PBS. At day 10 upon transfer, we evaluated the expansion of the antigen specific effector T cells in lymph nodes draining the immunization site. As shown in Fig 33 we observed a 2 fold higher expansion of OT-I cells in DEC205 immunized WKO mice than in WT animals. Moreover, OT-I cells frequency were higher in the control, not immunized group of WKO mice.

Previous results of our group showed that T cell activation induced by DEC205 DCs targeting (measured at day 3) is reduced in WKO mice due to impaired DC trafficking (Pulecio et al., 2008).
Thus, these results suggest that despite suboptimal T cell priming, effector cells survive better in the WKO host, which is in line with inducing effects of systemic type I IFN on CD8$^+$ T cells survival (Tough et al., 1996; Zhang et al., 1998; Marrack et al., 1999). This is further supported by the longer survival observed in the antigen independent context.
Discussion

Toll-like receptors (TLRs) are innate immune receptors that trigger inflammatory responses by various biological mechanisms such as cytokine production, recruitment of inflammatory cells, or activation of adaptive immunity. Although the innate immune system is designed to fight against infectious pathogens, excessive activation of TLR signaling pathways may lead to unwarranted inflammation with hazardous outcomes, including autoimmune diseases. Mechanisms that limit unrestrained activation of innate immune cells upon triggering of TLR are, therefore, of increasing interest as their potential in understanding autoimmunity.

This work provides for the first time a description of WASp, a regulator of actin cytoskeleton, in pDCs. PDCs are a small subset of DCs present in both human and mice specialized to rapidly secrete massive amounts of type-I interferons (type-I IFN) after viral infections. We show that type I IFN production is negatively regulated by WASp-dependent actin dynamics, both indirectly through cell extrinsic mechanism and in a cell autonomous fashion. The combination of extrinsic and intrinsic cellular mechanisms makes pDCs chronically activated and capable to constitutively release type-I IFN. These findings provide a new important clue to understand the development of autoimmune phenomena in WAS and suggest the inclusion of the syndrome in the list of type-I IFN diseases.

In the mouse model of WASp deficiency (WKO mice), we observed multiple signs of spontaneous pDCs activation. We reported an increased number of pDCs in secondary lymphoid organs of mutant mice that was in parallel with enhanced proliferation rate at steady state. Basal maturation profile of WKO pDCs further indicated a state of general constitutive activation, as MHC-II and co-stimulatory molecules CD40 and CD86 were expressed at significantly higher levels in lymphoid organs from WKO animals relative to wt mice. Activated phenotype of pDCs from WKO mice correlated with increased serum levels of IFN-α and ongoing transcription of IFN-α genes. This is in line with what reported in SLE patients, where increased basal levels of circulating IFN-α correlate with disease activity and severity (Bengtsson et al., 2000).

We found that besides enhanced expansion, pDCs isolated from WKO animals were more prone to undergo spontaneous apoptosis. This could fit with a recent study showing that acute TLR triggering during viral infections induces pDCs death and a marked reduction in cell numbers in a type-I IFN-dependent manner (Swiecky et al., 2011). In WASp mice, pDCs proliferate and die faster, however, the balance between increased proliferation and cell death is still in favor of the first most likely because stimulation is due to low levels of endogenous agonists as opposed to acute triggering. However, chronic pDCs activation at steady state correlated to inability to respond to exogenous stimuli by pDCs in adult mice. We found that chronically activated pDCs
become progressively refractory to further exogenous stimulation of TLR9, both in terms of co-stimulatory molecules expression and type-I IFN production. The inability of pDCs to make type-I IFN in response to a secondary challenge has already been described in the context of viral infections (Björck et al., 2004; Ito et al., 2006) and in SLE patients (Kwok et al., 2008). However, the exact mechanisms of TLR9 tolerance were not fully explained. Our results showed that CpG-induced IFN-α secretion was profoundly abolished in WKO mice, and this was specific of IFN pathway in pDCs, as inflammatory cytokines were equal in the two genotypes. This is unlikely to depend on differences in TLR9 expression as real time PCR revealed similar expression of the receptor in the two genotypes. We can exclude that unresponsiveness is only due to a higher apoptotic rate, as we have isolated specifically annexin-V negative cells in some experiments and still did not detect IFN-α transcription (data not shown). In addition, we found that about 40% of WKO pDCs underwent apoptosis after in vivo injection of TLR9 agonists. This frequency was higher relative to wt pDCs (~28%), but not sufficient to explain the total abrogation of IFN-α production observed in WKO cells (data not shown). Importantly, intracellular staining of pDCs in spleens upon CpG-A injection was done gating on live cells.

It is particularly intriguing that stimulated pDCs that fail to secrete IFN-α can still produce IL-6. Although we have not investigated this aspect, one possibility would be that continuous stimulation of TLR9 may selectively inhibit and/or deplete components of TLR9/IFN-α signaling platform. It is, in fact, a well known occurrence for cells that have been persistently stimulated with LPS to fail to respond to further re-stimulation because of down-regulation of crucial signaling proteins. Post-receptor signaling proteins that are altered in LPS tolerance include augmented degradation of IRAK, and decreased IRAK-MyD88 and TRAF6 association (Li et al., 2000; Medvedev et al., 2002). Activation of the negative regulator IRAK-M and suppressor of cytokine signaling (SOCS)-1 have been moreover found in LPS tolerance (Kobayashi et al., 2002; Kinjyo et al., 2002), suggesting the possibility that a persistent activation of TLR9 may associate to induction of genes that negatively regulate IFN-α responses.

Importantly, we have preliminary data showing that WKO pDCs are refractory to produce IFN-α also upon further stimulations of TLR7 (data not shown).

A prominent feature of WAS is the predisposition to develop pyogenic and opportunistic infections. Inner ear infections, such as bacterial pneumonia, sinusitis and meningitis have been frequently encountered in affected young males. Among the opportunistic infections, recurrent herpes simplex infection, *Pneumocystis carinii* pneumonia, and systemic varicella are common (Sullivan et al., 1994; Snapper and Rosen, 1999). Moreover, similarly to what observed in clinical WAS, it has been reported a failure of WKO mice to respond to pathogens, specifically to influenza A virus,
Discussion

*Streptococcus pneumoniae*, and *Mycobacterium bovis* (Andreansky et al., 2004). Whether exhaustion of the IFN-α pathway is playing a role in inefficient viral clearance in WKO mice is therefore a very important issue to address. Results obtained from future viral challenge experiments could provide important insight into the pathogenesis of WAS and the markedly increased incidence of viral infections in patients.

We found that two mechanisms contribute to unabated pDCs activation in WKO animals. We reported that sera from WKO mice older than 3 weeks (“adult mice”) contain extrinsic factors capable to induce activation of wt pDCs in terms of IFN-α production. The ability to induce IFN-α secretion correlates with the presence in these sera of anti-dsDNA autoantibodies, and it is significantly reduced when a pretreatment with DNAse is performed. These findings suggest that self DNA in complex with auto-antibodies (Humblet-Baron et al., 2007; Nikolov et al., 2010) are converted into pDCs inducers, similarly to what has been described in SLE (Barrat et al., 2005; Means et al., 2005). Importantly, we reported that sera from WKO mice younger than 3 weeks (“young mice”) did not induce pDCs activation nor displayed detectable autoantibodies, indicating that an inflammatory environment capable to activate pDCs establishes with aging in WKO mice. Because the treatment with DNAse did not completely abolish the IFN-α inducing capacity of sera from adult WKO mice, it is likely that other environmental factors participate in pDCs activation in WKO animals, such as RNA-containing ICs and heat shock proteins. Since it has been reported that phagocytic activity is markedly reduced in macrophages deficient for WASp (Lorenzi et al., 2000), we can hypothesize that all the material deriving from inefficient clearance of apoptotic/necrotic cells can represent a continue source of endogenous TLR9 agonists (Bave et al., 2000).

Second and most compelling, we demonstrated with different approaches that WASp deficient pDCs are intrinsically hyper-responsive to TLR9 triggering. We analyzed the responsiveness to CpG and endogenous triggers (sera from adult WKO mice) of pDCs from mice younger than 3 weeks. In “young mice” there are not endogenous triggers of pDCs, and an inflammatory environment is not still established, as demonstrated by the maturation profile of WKO pDCs that isolated from young animals did not display phenotypic activation. We found that after CpG stimulation, young WKO pDCs are not only responding but they are even hyper-responsive in terms of both, regulation of maturation markers expression, and type-I IFN production. In addition, pDCs isolated from young WKO animals respond more vigorously than pDCs from young wt mice to stimulation with sera derived from adult WKO animals. These data indicate a two-levels mechanism implicated in constitutive WKO pDCs activation: on one side an intrinsic lower threshold, on the other the presence of endogenous TLR9 inducers. We definitely
demonstrated cell-intrinsic hyper-reactivity using WASp depleted pDCs, a setting that completely excludes inflammatory/environmental factors. We used in parallel two siRNAs to silence WASp expression, with a silencing capability of approximately 70% and 55%. Interestingly, we found that a reduction in protein expression of 55% was sufficient to unveil an hyper-reactive phenotype, suggesting that cells with missense mutations, that lead to decreased protein expression, may manifest the phenotype. Inducing a general block in actin polymerization by chemical inhibitors, such as cytochalasin-D, caused a large increase in IFN-α release upon CpG-A stimulation. Consistently to what observed in WASp-depleted cells, enhanced responses were restricted to IFN-α in pDCs as inflammatory cytokine production and activation of conventional DCs were not affected. These findings are really interesting, because highlight for the first time a role of actin cytoskeleton in negative regulation of type-I IFN in pDCs and indicate that indeed WASp deficiency perturb IFN-α production via its actin regulatory properties.

Inhibition of IFN-α production uncoupled from inflammatory cytokine production was previously reported in cells lacking proteins required for translocation of IRF-7 to the nucleus (Gotoh et al., 2010; Guiducci et al., 2008) or that interfere with TLR9 endosomal trafficking (Sasai et al., 2010). Yet, to our knowledge this is the first report describing enhanced type-I IFN production as a consequence of an altered endocytic pathway. Our data strongly suggest that WASp is required for actin assembly in early endosomes (EEs) and for subsequent segregation. Although we have not identified the exact sequence of events that cause endosomes enlargement and CpG accumulation, our findings, in addition to those from many other research groups, help hypothesize the mechanism by which WASp controls this process. Firstly, we found that tyrosine phosphorylation of WASp is induced following CpG-A stimulation of pDCs. Phosphorylation of WASp has emerged as an important physiologic regulator of WASp activity. Particularly, a single WASp tyrosine phosphorylation site (Y291), is found to be a target for non receptor kinases, such as Btk and Fyn, the latter one belonging to Src family kinase (SFKs) (Baba et al., 1999; Badour et al., 2004). Phosphorylation of this residue activates WASp through disruption of the autoinhibited conformation and is critical for multiple WASp effector functions, including an efficient actin polymerization (Badour et al., 2004). Our findings report for the first time that WASp is implicated in signalling downstream TLR9 and let us to speculate a role for WASp as a nexus for controlling actin reorganization in pDCs stimulated with CpG. Furthermore, our data illustrate that activation of TLR9 by CpG-A DNA triggers tyrosine phosphorylation of SFKs, and two proteins that, in turn, are known to be tyrosine phosphorylated by SFKs, the cytoskeletal protein paxillin and the non receptor protein-tyrosine kinase Pyk2. Paxillin is an adaptor protein that functions as a scaffold to recruit
specific proteins, included Pyk2. Several studies have revealed a correlation between Tyr-
phosphorylation-mediated activation of these two protein and formation of complexes promoting
actin rearrangements and membrane dynamics in TLR-stimulated hematopoietic cells (Suen et al.,
1999; Williams and Ridley, 2000). Based on these findings, we propose that key biochemical events
involving Tyr-phosphorylation may control the formation of complexes that govern cytoskeletal
changes required for CpG to promote specific WASp-mediated functions in pDCs. CpG-A
stimulation of pDCs correlated with accumulation of WASp in EEs, strongly supporting a role of
WASp in remodeling of early endosomal actin during TLR9-mediated pDCs activation. In addition,
we reported that lack of WASp induced profound alteration of cell architecture and enlargement of
EEs with appearance of irregular tubular endosomal structures and large aggregates of CpG-A
spread in the cytosol. This scenario resembled what observed in pDCs where depletion of cortactin,
an interactor of WASp, and actin depolimerizing drugs were shown to induce block of EE
segregation and inhibition of maturation/translocation of EEs toward the perinuclear region (Ohashi
et al., 2011). The process of membrane remodeling for the endosomal maturation pathway,
including tubulation and segregation activities, is of pivotal importance, as it sort internalized
molecules to degradation, recycling or other intracellular organelles in this way controlling the
signaling fate.

Previous studies have reported the strict correlation of type-I IFN production in pDCs and spatio-
temporal regulation of endocytic pathway. PDCs retain the TLR9-bound CpG-A and MyD88-IRF-7
complex in early endosomes for long periods, and this is required for a robust IFN response.
Consequently, following trafficking to late endosomes/lysosomes irrevocably terminates the
signaling, and disruption of this process would be expected to prolong signaling of TLR9 in EEs
with sustained IFN induction. It was logical for us to hypothesize that WASp may nucleate actin on
CpG-containing EEs, and that this branched actin network play an essential role in controlling
fission of these organelles. All a series of studies strongly support this hypothesis. First, N-WASp-
motivated actin polymerization is found to be required for the internalization of clathrin-coated pits
during clathrin-mediated endocytosis (Takenawa and Suetsugu, 2007), the pathway used by CpG-A
to enter cells (Latz et al., 2004). Second, actin polymerization mediated by N-WASp is required for
dynamin-mediated fission of tubules induced by the overexpression of F-BAR/ECF proteins (Itoh et
al., 2005; Tsujita et al., 2006). Importantly, the role we propose for WASp in mediating endosomal
fission is analogous to the role of WASH, a member of WASp family that critically induces
endosome sorting by facilitating tubule fission via Arp2/3 activation (Derivery et al., 2009; Gomez
and Billadeau, 2009). Taken together, these reports and our results, are actually consistent with a
model in which WASp may restrain TLR9 signaling by driving local actin polymerization at EEs,
thus generating mechanical forces that promote tubule scission (fission) and maturation into late organelles, preventing excessive IFN response. It would be interesting to evaluate whether the increased responses to TLR9 triggering recently reported in WASp null B cells (Becker-Herman et al., 2011) is linked to a similar alteration of endocytic organelles.

The relevance of our findings in the mouse model of the disease has been validated in WAS patients. Analysis of gene expression on PBMC of pediatric WAS patients with high clinical score showed increased expression of IFN-α genes in 85% of patients analyzed. Moreover, we observed in this cohort of patients significantly higher levels of a group of genes known as downstream events after type-I IFN stimulation. This pattern of up-regulated type-I IFN/type-I IFN inducible genes is characteristic of viral infections, SLE, and several other autoimmune disease (Pascual et al., 2010), and it is known as type-I IFN signature. SLE patients display an IFN signature in PBMC, especially early in the disease process, even in absence of measurable serum levels of IFN-α (Bennet et al., 2003; Baechler et al., 2003). We also performed the analysis in spleens of affected young WAS patients of the protein level of MxA, one of the most prominent proteins induced by type-I IFN. The immunohistochemical analysis revealed in 5/8 patients high expression of MxA, definitely demonstrating a systemic exposure to this cytokine. We also reported a reduced frequency of pDCs in the peripheral blood of our cohort of pediatric WAS. This is, again, in line with what reported in lupus, where the frequency of pDCs in blood is reduced, but activated IFN-α-secreting cells can be found in peripheral tissue (dermal lesions and in noninflammatory skin) (Blomberg et al., 2001). However, this finding is incongruent with the increased numbers of pDCs observed in WKO mice. This discrepancy may be explained by the exposure of WAS patients to repeated infections and/or by establishment of an inflammatory environment that are known to associate with massive recruitment in peripheral tissues and induction of apoptotic death of pDCs (Duan et al., 2004; Blomberg et al., 2001; Swiecky et al., 2011). Moreover, most of the WAS patients are treated with corticosteroid to control eczema and autoimmune manifestations, a second factor that can contribute to pDCs depletion. Prednisolone, for instance, an effective therapeutic agent in WAS, has been found to induce apoptosis of pDCs with consequent reduction in their circulating numbers (Boor et al., 2006). Together, the findings in WAS patients resemble the features invariably observed in SLE patients, where the pathogenic role of exaggerated production of type-I IFN by pDCs has been firmly established (Bennet et al., 2003; Blomberg et al., 2001; Cederblad et al., 1998). In light of our results, it would be interesting to investigate whether in SLE patients there are polymorphisms in actin-related gene that may predispose to SLE by increasing the ability of pDCs to release type-I IFN upon activation.

Our work is the first report that describes IFN-α alterations in WAS. This is of great
relevance as increased levels of type-I IFN may be a crucial initiating factor for the development of autoimmune phenomena. A plethora of literature describing the development of anti-nuclear antibodies, lupus symptoms and even full-blown SLE in human treated with recombinant IFN-α is available (Ronnblom et al., 2003). It is well known that IFN-α can induce loss of B and T cell tolerance directly by promoting proliferation of autoreactive B cells and T cells (Jego et al., 2003; Le Bon et al., 2006 a; Le Bon et al., 2006 b). Moreover IFN-α diverts the tolerogenic properties of immature DCs by inducing their activation that in turn promote expansion of self-reactive T and B cells (Blanco et al., 2000; Zhu et al., 2005; Le Bon et al., 2001; Litinskiy et al., 2002; Kolumam et al., 2005; Le Bon et al., 2003 ). We have undertaken an initial analysis in the mouse model aimed at characterizing signs of dysregulated IFN-α production. We found that conventional DCs isolated from WKO mice show higher basal levels of maturation markers than their normal counterparts and hyper-responsiveness upon TLR4 triggering in terms of both expression of co-stimulatory molecules and inflammatory cytokine production. Importantly, the fact that using a neutralizing antibody to IFN-α abrogated the enhanced maturation of wt cDCs induced by WKO surnatants provides a strong evidence that WKO cells-derived type-I IFN plays inducing effects on cells of immune system. In parallel, we reported a more activated basal phenotype in B cells from WKO mice, that may be explained as a consequence of direct activating effects of circulating type-I IFN or IFN-induced cDCs. Moreover, we reported that spontaneous activation and further enhanced maturation response after TLR4 triggering of WKO cDCs was reflected in enhanced ability to activate antigen specific CD4+ T cells and higher release of IL-6 upon interaction with T cells. We further investigated the Th fate of wt CD4+ T cells in light of the prominent role of IL-6 in promoting differentiation toward Th17 cells, a third subset of T helper cells that play key roles in inflammation and tissue damage both in animal models and human autoimmune diseases. Preliminary data indicate a strong polarization toward Th17 cells induced by WKO hyper-activated cDCs, suggesting a potential direct link between altered pDCs/IFN-α axis, Th17 development, and autoimmune disorders present in WAS. IFN-α, and IFN-induced cDCs are also known to exert several effects on CD8+ T cells particularly relevant to the pathogenesis of autoimmunity, including increased activation, survival and cross-presentation (Tough et al., 1996; Zhang et al., 1998; Marrack et al., 1999). Our initial evidences report that immunization with αDEC205-OVA of WKO leads to enhanced expansion and prolonged survival of wt CD8+ T cells relative to wt animals. Importantly, we found a major expansion and prolonged survival of OT-I cells also in not immunized group of WKO mice. Our group has previously shown that initial T cell activation induced by DEC205 DCs targeting, measured at day 3, is reduced in WKO mice due to impaired DC trafficking (Pulecio et al., 2008). Thus, these last results suggest that despite suboptimal T cell
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priming, effector cells survive better in the WKO host, which is in line with inducing effects of systemic type I IFN on CD8+ T cells survival. This is further supported by the longer survival observed in the antigen independent context. The fate of T cells primed in conditions that are known to induce T cell deletion in WT mice, i.e., steady state targeting of DCs in the absence of adjuvant, is an interesting question to be address in WKO mice. Based on the above data we would predict that IFN-α conditioned DCs in WKO mice will not be able to induce peripheral tolerance in this model. Finally, we observed tangible signs of autoimmunity in WKO animals. These were proteinuria, splenomegaly, and lymphocytic sialoadenitis, all classic symptoms of a generalized chronic inflammation. All together these data are consistent with an IFN-induced chronic inflammatory environment. However, future experiments will be necessary to definitely demonstrate the direct association between dysregulated production of IFN-α and the onset of inflammatory phenotypes observed in WKO mice. Experiments conducted in WKO animals backcrossed to mice KO for the type I IFN receptor will be decisive in this sense.

Although a comprehensive understanding of the way in which WASp mechanistically regulates endosomal actin dynamics requires further investigation, and even a direct association between the inflammatory environment present in WKO mice and type-I IFN is still lacking, our findings allowed us to formulate a working model.

1) It is plausible to think that because of the phagocytosis defect of macrophages in WKO animals material deriving from not properly cleared apoptotic/necrotic cells start to accumulate representing a first source of possible endogenous pDCs triggers. 2) Lack of WASp causes alterations of
endosomal actin with resulting enlargement of early endosomes. This predispose pDCs to induce massive IFN-α release in response of a first wave of endogenous triggers. 3) Type-I IFN induce maturation of both, pDC and cDCs. 4) Activated cDCs may tilt the fate of autoreactive T lymphocytes that have escaped central tolerance to activation. Mature cDCs may also activate cytotoxic CD8+ T cells that generate nucleosomes which can be taken up by IFN-induced cDCs. 5) IFN-induced cDCs activate CD4+ T cells, and produce higher levels of IL-6 promoting polarization of CD4+ T cells toward Th17 subset. 6) Together with IL-6, type-I IFN promotes activation of B cells, and differentiation of mature B cells into plasma cells predisposing to autoantibodies production. Self nucleic acids-containing ICs may activate B cells through the co-engagement of BCR and TLRs. PDCs secrete more IFN through the co-engagement of FcγR and TLRs. This may result in an amplification of a pathogenic loop, where increased levels of autoantibodies are produced generating more interferogenic ICs that sustain the type I IFN production. Elevated levels of circulating IFN-α may result in desensitization pDC to further TLR stimulation (7). This last event may play a role in WAS-associated immunodeficiency.

In conclusion, this is the first report that demonstrates altered TLR9 signaling in WASp-deficient pDCs. These observations add a new layer of complexity to our understanding of the pathophysiology of this disease, and raise important considerations about the treatment of patients with autoimmune phenomena. Finally, the implications of this study go beyond the pathophysiology of WAS, introducing actin as a new player in the complex scenario of the negative regulation of IFN-α production in pDCs.
5 REFERENCES


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