4. General Introduction

4.1. Influenza virus infection

Recent outbreaks of highly pathogenic influenza A virus infections in poultry and humans (“birdflu”) and the potential threat of influenza pandemics with high mortality demand a better understanding of the pathogenesis of this disease. In addition, novel vaccines and treatments options are necessary to prevent or mitigate large-scale outbreaks of influenza virus infection. The virus mainly attacks cells of the upper respiratory tract including the nose, throat and the bronchial tree. The disease is characterized by the sudden onset of high fever, sore throat, rhinitis, cough and headache. While infected individuals recover within a week, influenza poses a serious risk for the very young and elderly, potentially resulting in pneumonia and death. Estimates by the World Health Organization (WHO) depict 3-5 million severely ill, and 250,000 to 500,000 deaths related to influenza per year. To date, vaccination with inactivated viruses or subunits of viruses are only partially effective. After a primary infection or vaccination with a certain virus strain, the epitope-specific neutralizing antibodies produced by B cells protect against reinfection with the same or closely-related viruses. However, the protection is reduced or lost for infection with variant viruses caused by genetic changes due to mutation (antigenic drift) and reassortment (antigenic shift) in influenza viruses. Consequently, vaccine compositions need to be adjusted annually to include the most recent circulating influenza strains. Therefore, research has started to explore other arms of the immune system including innate immune cells involved in the first line of defense, as well as T cell-mediated immunity. Key participants in the initiation of the immune response are innately existing components and cells such as macrophages and dendritic cells (DCs). In particular, a subset of DCs, the plasmacytoid dendritic cells (pDCs), is thought to play a major role in the antiviral defense. In addition to forming a network in cross-talking to and activating other antiviral mediators, i.e. natural killer (NK) cells, DCs also represent an important initiator of the T cell- and B cell-mediated adaptive immunity that

results in the resolution of infection and protection against reinfection. The overall aim of this thesis was to further our understanding of how innate immune cells regulate anti-influenza immune responses.

4.1.1. Influenza virus A and its life cycle

Influenza A virus belongs to the family of Orthomyxoviridae, which are enveloped viruses with a segmented single-stranded RNA (ss-RNA) genome [1]. For influenza A viruses, subtyping is based on the antigenicity of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (Figure 1A). HA and NA are responsible for virus attachment and penetration into cells as well as release of progeny virus from the infected cell. Influenza viruses encode also for M2 (integral membrane ion-channel protein), proteins comprising the ribonucleoprotein complex NP (nucleoprotein associated with RNA), and the polymerase proteins PA, PB1 and PB2. The matrix protein M1 is associated with both the ribonucleoprotein and the viral envelope and underlies the lipid bilayer. NS1 and NS2 represent non-structural proteins.

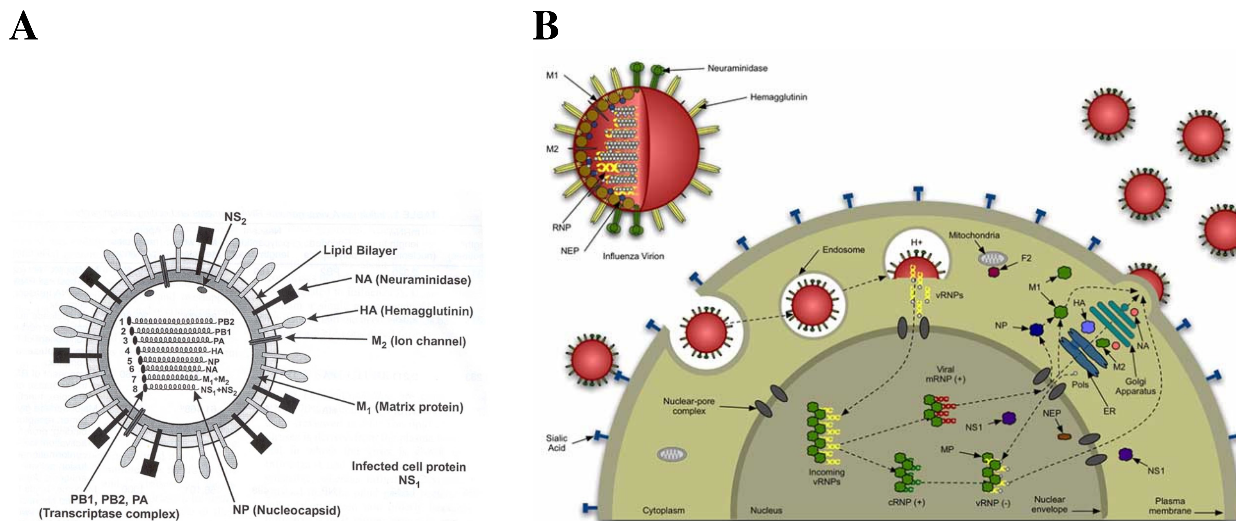

Figure 1: Schematic diagram (A) of the structure of an influenza A virus particle and (B) of the life cycle of influenza virus.

(taken from www.reactome.org)

In humans, the HA surface glycoprotein mediates virus binding to host-cell receptors containing sialyloligosaccarides terminated by N-acetyl sialic acid linked to galactose by an α2,6 linkage (NeuAcα2,6Gal). Mice were shown not to express the α2,6-linked sialic acid receptors, but share with humans the expression of α2,3-type receptors [2]. Of note, avian viruses preferentially recognize NeuAcα2,3Gal linkages [3]. The specificity of human trachea, which contains mainly α2,6-linked carbohydrate chains, is a key determinant in restricting the transfer of influenza virus directly from avian species that contain mainly α2,3 linkages on epithelial cells in the trachea.

After binding, the virus is internalized by endocytosis, followed by the fusion between the viral envelope and the endosomal membrane, which allows for delivery of the viral ribonucleoproteins into the cytosol and further transport into the nucleus. Among the RNA viruses, influenza virus is unique, as all of its RNA synthesis – transcription and replication – takes place in the nucleus of the infected cell (Figure 1B). In the nucleus, the viral negative-strand RNA (vRNA) serves as a template for the synthesis of capped, polyadenylated viral messenger RNA and of full-length positive-strand RNA or complementary RNA (cRNA). The cRNA serves as a template for the synthesis of new vRNA molecules. Viral RNA polymerase enters the host cell nucleus with the viral RNP complex and catalyzes all three of these reactions. For export, vRNA is packaged into ribonucleoprotein (RNP) complexes containing two viral proteins, NP and M1. Following protein synthesis, the three viral integral membrane proteins, HA, NA and M2 enter the host endoplasmic reticulum (ER), where all three are folded and HA and NA are glycosylated. HA, NA and M2 are transported to the Golgi apparatus where cysteine residues on HA and M2 are palmitoylated. All three proteins are directed to the virus assembly site on the apical plasma membrane. Influenza virus buds preferentially from lipid raft microdomains of virally infected cells to release progeny virus into the surrounding tissue.

4.2. Immunological resistance to influenza virus infections

Specific immune mechanisms have evolved that recognize and, in most cases, eliminate infectious agents. Specialized classes of immune cells contribute to responses resulting in protection against a possible reinfection. The immune response occurs in three phases summarized in Figure 2.

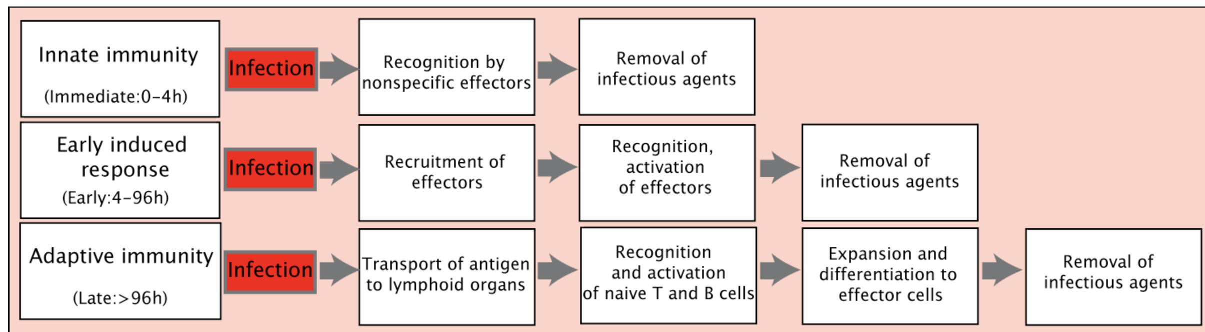

Figure 2: Three phases of an immune response against infection.
The first two phases rely on recognition of pathogens by innate receptors and occur early after infection. The adaptive immunity involves antigen-specific receptors and occurs at later times, because the rare T and B cells specific for an antigen must undergo clonal expansion and acquire effector functions before participating in the fight against the invading microbe. (Adapted from Janeway et al., Immunobiology, 5th edition)

In the first line of defense, cells of the innate immune system such as DCs and macrophages make contact with the invading virus. Tissue-resident cells can rapidly recruit other early effectors such as granulocytes and NK cells. However, innate immune cells are unable to confer antigen-specific actions and immune memory. These are tasks of cells of the adaptive arm, T and B lymphocytes. When activated, $CD8^+$ T cells differentiate into cytotoxic killer cells (CTL) that destroy virus-infected target cells. $CD4^+$ T cells differentiate into T helper (Th) cells. Th cells activate other immune cells as well as B cells that become plasma cells, which secrete antibodies. T and B lymphocytes also give rise to antigen-specific memory cells that can respond faster and more effectively than naïve cells in a secondary

infection. Dendritic cells are key players in an immune response as they represent an important link between the innate and the adaptive division in immunity.

4.2.1. Recognition of respiratory viruses

The lung continuously samples the air containing microbes and other particles, and can thus be a portal for pathogen entry. Upon infection, the presence of viral particles in infected epithelial cells and stromal cells of the airways induces an inflammation that signals “danger and presence of harmful (non-self) material” to the surrounding tissue. Inflammation is caused by chemical factors, such as cytokines, released from injured or infected cells. Several innate mechanisms exist to detect invading pathogens that have crossed the natural physical barriers of the body [4]. First, soluble factors, termed collectins can bind sugar molecules present on the pathogen’s surface [5]. Members of this family are mannose-binding lectin and C1q in the blood and surfactant A in the alveolar fluid of the lungs. Activation results in a pathway known as the complement cascade that will help to destroy invading pathogens by cell lysis. The second way of detection occurs by evolutionary conserved pathogen-associated molecular patterns (PAMPs) and their binding to recognition receptors (PRR) known as Toll-like receptors (TLRs). TLRs are expressed by several innate immune cells including DCs and macrophages as well as neutrophils and NK cells [6]. The main ligands recognized by different TLRs, and their origins are summarized in Table I.

In antiviral immune responses, there are three main Toll-like receptors triggered by viral components, TLR3, TLR7 and TLR9. These receptors share the feature of recognizing nucleic acids and are expressed – unlike all other TLRs, which are located on the cell surface – in an endosomal compartment. As key sentinel cells, macrophages and DCs are the main cells expressing these receptors.

Toll-like receptors and their ligands		
Receptor	*Ligand*	*Pathogen*
TLR1	**Triacyl lipopeptides**	**Bacteria**
TLR2	**Peptidoglycans, Zymosan**	**Bacteria and Fungi**
TLR3	**ds-RNA**	**Viruses**
TLR4	**Lipopolysaccharide**	**Bacteria**
TLR5	**Flagellin**	**Bacteria**
TLR6	**Diacyl lipopeptides, Zymosan**	**Bacteria and Fungi**
TLR7/8	**ss-RNA**	**Viruses**
TLR9	**CpG-containing DNA**	**Bacteria and Viruses**
TLR10	***unknown***	***unknown***
TLR11	**Profilin**	**T. gondii, uropathogenic Bacteria**
TLR12	***unknown***	***unknown***
TLR13	***unknown***	***unknown***

Table I. Excerpt of Toll-like receptors and their main ligands.

Additional components of a virus-detection system deserve to be mentioned [7]. Receptors for ds-RNA, a byproduct during viral replication, have been identified inside in the cytosol of certain cells, a prominent member of which is protein kinase ds-RNA-dependent (PKR). Moreover, caspase recruitment domain (CARD)-containing helicases such as RIG-I, MDA5 and NOD1 and 2. Members of the PYRIN- (Nalps) family have also been implicated in viral recognition although the precise nature of their function remains unknown [8, 9].

4.2.2. Dendritic cells: Link between innate and adaptive immunity

DCs comprise a family of professional antigen presenting cells (APCs) [10, 11]. In mice, at least five different subsets have been identified. All DCs express CD11c (the integrin α_x-chain), and are further subdivided depending on their expression of CD11b (the integrin α_M-chain), CD4, CD8 and CD205. Depending on their origin from certain hematopoietic

progenitor cells, DCs have also been classified into lymphoid ($CD8^+$ DCs) and myeloid DCs ($CD11b^+ CD8^-$ DCs). pDCs have recently been added to the list of DCs as a separate subset. They are characterized by a very distinct phenotype as they express intermediate level of CD11c and co-express the B cell marker B220. PDCs will be described in more detail below (see 4.3.). Based on their migratory properties, another division of DCs has been proposed, i.e. migratory versus (lymphoid) tissue-resident [12]. A typical migratory DC is the Langerhans cell, which is found in the skin and the skin-draining lymph nodes (LNs). When reaching the LNs, migratory DCs have a mature phenotype, while lymph node-sessile DCs are immature [13].

DCs stand guard in most tissues in an 'immature' state fully equipped to take up particles and microbes by different processes including phagocytosis, macropinocytosis, and receptor-mediated endocytosis. In the lungs, an extensive network of DCs has been shown to reside within the mucosa, the conducting airways, the alveolar lumen, and the connective tissues surrounding the blood vessels and pleura [14, 15]. Considerable heterogeneity exists in DCs residing in these different compartments [16]. In the absence of inflammation, DCs or their precursors are constantly recruited from the blood into the lung. However, it is unclear whether they are recruited as precursors in a differentiated form or whether the pool of intrapulmonary DCs undergoes renewal through local differentiation. PDCs are absent from the airway compartment of naïve mice, however they reside in lung parenchyma under non-inflammatory conditions [17, 18].

A hallmark of DCs is their capacity to act as APCs with two main functions: 1) Antigen capture, processing and transport, and 2) initiation of adaptive immune responses [10, 19]. In the steady-state, experiments using instillation of inert fluorescent molecules into the airways of mice have suggested that small numbers of DCs may constantly travel from the lungs to the draining cervical (cervLNs) and mediastinal lymph nodes (medLN) transporting antigen [20-23]. This process may be part of a system of (self-) tolerance maintenance or sustaining regulatory T cells that may form constant immunesuppression against harmless antigens.

Interestingly, a special role in preventing asthmatic symptoms after the inhalation of an harmless antigen has been ascribed to pDCs [17, 24].

Under inflammatory conditions, DCs that captured antigen undergo a remarkable transformation into mature DCs. During this transition, DCs remodel their migratory behavior and leave the tissue traveling via afferent lymphatics to the lymph nodes in order to encounter naïve antigen-specific T cells (Figure 3) [25].

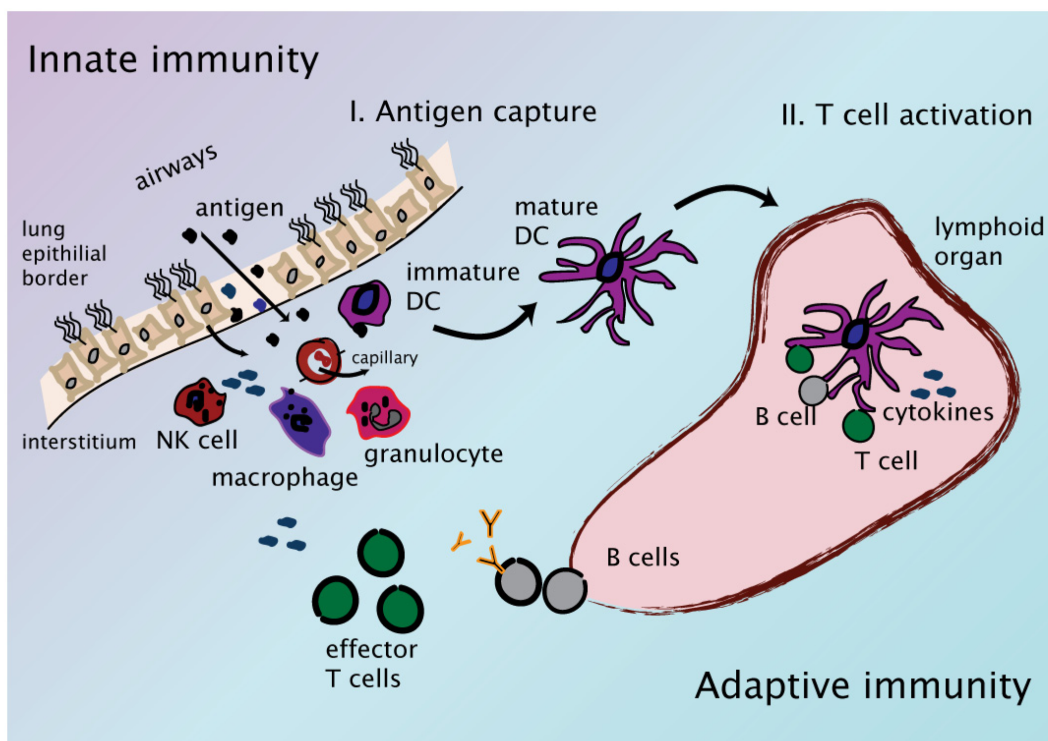

Figure 3: Paradigm of innate and adaptive immunity following lung infection.

Pathogen invasion initiates a cascade of processes that control a coordinated immune response. After antigen uptake by pulmonary dendritic cells and macrophages, their maturation and migration to lymph nodes result in encounter of antigen-specific T cells, which are subsequently activated. After differentiation, effector T cells migrate to the site of infection and combat together with NK cells infected cells and microbe progeny. B cells are activated to produce antibodies, which protect against reinfection with the same virus.

Pathogen-activated DCs induce activation of T cells in three ways; Determinant 1: MHC-peptide complex, Determinant 2: Costimulatory signal, Determinant 3: Cytokine milieu (for review see [26]). During maturation DCs degrade some of the ingested particles into peptide fragments, which are loaded onto major histocompatibility complex (MHC) molecules and presented on the cell's surface. In the lymph node, DCs are sampled by naïve T cells, each of which express a T cell receptor (TCR) specific for a certain antigen. In the presence of cognate antigen, DCs and T cells rapidly form a tight contact, called the immunological synapse [27]. The interaction of the TCR with MHC-peptide complexes on the DC represents the primary activation signal for T cells. In addition, DCs upregulate MHC class II molecules and costimulatory molecules such as CD40, CD80 and CD86 providing a second signal binding to the complementary receptors on the T cell surface, including CD28, ICOS (inducible costimulator ligand), CD40 ligand (CD40L), Ox-40 and 4-1BB (CD137). These interactions are necessary for clonal expansion, induction of differentiation into effector T cells and avoidance of T cell anergy (non-responsiveness) or apoptosis (activation-induced cell death). The activated T cell produces interleukin-2 (IL-2) and upregulates the expression of CD40L to provide a positive feedback signal to the DC. DCs in LNs also interact and directly stimulate B cells [28]. The program of DC maturation results in further acquisition of effector functions such as the production of cytokines IL-1, IL-6, TNF-α, IL-12 or IL-4. Cytokines co-induce T cell differentiation from naïve $CD8^+$ and $CD4^+$ T cells to cytotoxic effector $CD8^+$ T cells (CTL) and $CD4^+$ T helper cells (Th cells) and determine the degree of polarization of Th cells (see below).

4.2.3. Adaptive immunity to respiratory virus infections

Lung infections demand immune responses to be targeted to the respiratory tract, and it has been well established that T lymphocytes, especially $CD8^+$ CTL, play a key role in clearance of viral pathogens from the lung. The generation, regulation and function as well as the migratory pathways of effector and memory T cells in response to influenza infections have been the subject of intense investigations (for review see [29]).

Upon encounter of mature APCs presenting viral peptides in the lung-draining LNs, naïve T cells become activated, proliferate and differentiate into effector cells. During this process, they downregulate lymph node homing molecules such as CD62L (L-selectin) and the chemokine receptor CCR7 and upregulate molecules (CD11a, α_1 and α_4 integrins, CD43, CCR5) that increase their capability to migrate to sites of inflammation, including the lungs [30]. Effector T cells acquire the capacity to produce antiviral cytokines, predominantly IFN-γ and TNF-α. CTL can also secrete perforin and granzymes that lyse infected target cells via cytolytic mechanisms [31]. In addition, B cells produce anti-influenza-specific antibodies that help in clearance of the virus. Usually, influenza virus is cleared by day 9 or 10 post infection (p.i.).

Effector CD4$^+$ T cells come in three flavors, Th1 cells, predominantly expressing IFN-γ, Th2 cells, secreting IL-4, IL-5, IL-13, and a newly discovered lineage, Th17, that secretes mainly IL-17 [32]. Depending on the original encounter and the milieu of infection, DCs instruct T cells during activation to commit to Th1 cells by production of IL-12 and IL-18, or to Th2 cells by secreting IL-4 [26]. IL-17-producing cells are generated in the presence of IL-23, and are mainly associated with autoimmune diseases. However, increased IL-17 levels have been observed in virus-infected lungs in combination with allergic hyperresponsiveness [33]. In antiviral immunity, polarization to Th1 cells and development of CTL is predominant. Type 1 cytokines are crucial in augmenting the phagocytic functions of macrophages and neutrophils to support the destruction of virus-infected cells. IFN-γ exhibits a central role in immunity to many viral and also bacterial lung infections due to its cytotoxic activity. Thus, it has been shown that IFN-$\gamma^{-/-}$ mice display increased mortality and are more susceptible to infections with *L. pneumophilia, Klebsiella pneumoniae and M. tuberculosis* [34]. One of the major functions of CD4$^+$ effector T cells in addition to the release of effector cytokines is to help B cell maturation and promote their antibody secretion. Another key activity of CD4$^+$ T cells is their help in supporting optimal CD8$^+$ T cell memory differentiation [35].

The contribution of memory $CD8^+$ and $CD4^+$ T cells to protection against reinfection with serologically distinct viruses (heterosubtypic immunity) has been corroborated. In this type of immunity, T cells can target internal virus antigens that are shared between heterologous viruses (cross-reactivity). Thus, in contrast to antibodies that can only recognize subtype-specific surface proteins of the virus, which are similar between homologous but not heterologous viral strains, this form of immunity may provide protection (for review see [36]). However, the role of T cells in secondary immune responses is still incompletely understood, and is subject of intense investigations.

In addition to T cell mediated immunity, recovery of mice following influenza virus infection has been shown to depend on B cell responses, as B-cell deficient μMT mice showed significantly reduced survival compared to wild-type mice [37]. Antibodies can neutralize the virus, inhibit virus-attachment to cells and decrease virus progeny to infect other target cells. B cells make both Th-dependent and -independent contributions to the control and resolution of influenza virus infection. Although humoral immunity provides good protection against secondary infection with the same virus, it is ineffective against heterosubtypic viruses.

4.3. Plasmacytoid dendritic cells in immunity

It has been known for a long time that a specific subpopulation of leukocytes exhibits the remarkable capacity to produce high amounts of type I interferon after contact with virus. In 1999, human plasmacytoid dendritic cells from peripheral blood and lymph nodes were identified as this enigmatic type I-interferon-producing cell [38, 39]. In 2001, three independent groups reported the identification of the murine counterpart, which was shown to share most of the morphological, phenotypic and functional characteristics of the human pDC [40-42]. Recent studies have begun to elucidate mechanisms of their activation and functions. An increasing number of studies indicate a crucial role for pDCs in infectious diseases, allergic responses, control of T cell tolerance, autoimmune diseases and cancer.

4.3.1. Phenotype and development of plasmacytoid dendritic cells

In contrast to CD11c^{high} myeloid dendritic cells (mDCs), immature pDCs exhibit a plasma-like, round morphology (Figure 4). In mice, immature pDCs are defined as B220^{+} CD11c^{int} Ly6C^{+} CD11b^{-} CD4^{+} cells. Human pDCs exhibit a slightly different phenotype, i.e. CD11c^{-} IL-3Rα^{+} ILT-3^{+} CD45RA^{+} CD4^{+} [43, 44]. Additional antibodies in mice recognize pDC-specific surface molecules such as 120G8 [18], 440c [45] and mPDCA-1 [46] in the steady state. However, other cells upregulate these markers upon activation during inflammation. Whereas the antigens for 120G8 and mPDCA-1 remain to be characterized, the 440c antigen was recently identified as Siglec-H [47].

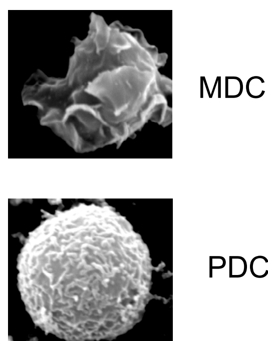

Figure 4: Electron micrographs from freshly isolated human CD11c^{hi} CD123^{-} myeloid DC (MDC) and CD11c^{-} CD123^{+} plasmacytoid DC (PDC).

(Courtesy of Dr. L. L. Cavanagh)

PDCs develop in the bone marrow (BM), and then distribute throughout the body via the blood stream. Recent studies could show that the transcription factors interferon consensus sequence-binding protein (ICSBP) and Ikaros play a critical role in the regulation of pDC development [48, 49]. The cytokine granulocyte-stimulating factor (G-CSF) promotes pDC mobilization from the bone marrow [50]. Another crucial factor for the development of pDCs from hematopoietic precursors is the cytokine Flt-3 ligand (Flt-3L, FMS-related tyrosine kinase 3 ligand). Flt-3L-deficient mice have highly reduced numbers of pDCs as well as mDCs, and injection of recombinant Flt-3L into mice can increase both cell types [41, 51]. PDCs can be expanded from BM in *in vitro* cultures by addition of Flt-3L.

The precise lineage relationship of pDCs is still unclear. Whereas some studies show that pDCs can be generated from either common lymphoid precursor (CLP) or common myeloid progenitor (CMP) cells [52, 53], another study suggests that pDCs arise exclusively from CMPs [54]. Within the BM of mice, a putative pDC precursor has been described that is $B220^+$ $CD11c^{int}$ $Ly49Q^-$, whereas in the periphery, all pDCs are $Ly49Q^+$ [55, 56]. Others claim that pDCs arise as two different subsets in the BM with distinct abilities and cytokine production patterns [57]. Using RAG1/GFP knockin mice, Pelayo et al. described a GFP^+ (pDC1) population that produced higher levels of IFN-α, IL-6 and TNF-α as compared to the GFP^- (pDC2) population that produced predominantly IL-12 and IFN-γ, and which also elicited better T cell proliferation [57]. Interestingly, they found these subsets not only in the BM but also in the spleen of RAG1/GFP knockin mice, indicating that both populations are stably present in the periphery of mice.

4.3.2. Activation of plasmacytoid dendritic cells

4.3.2.1. Expression of Toll-like receptors and recognition of nucleic acids

The ability of pDCs to switch to type I interferon-producing cells depends on the recognition of microbial components. PDCs express a specific subset of Toll-like receptors, namely TLR7 and TLR9 [58, 59]. The response of pDCs to viral components has been mainly deciphered by the use of TLR7- and TLR9-deficient mice. Whereas pDCs from $TLR9^{-/-}$ mice respond to RNA viruses such as influenza virus, TLR9 is required for recognition of DNA viruses such as herpes simplex virus (HSV) and murine cytomegalovirus (CMV) [46, 60, 61]. On the other hand, TLR7 mediates the response to ss-RNA viruses [62-64]. In contrast to many other TLRs that are located on the cell surface, TLR7 and TLR9 (and also TLR3) are contained intracellularly in the endosomal compartment [65]. It is possible that this localization prevents recognition of self nucleic acids but allows access to viral genomes after virus internalization and delivery to the lysosomal compartment, where the released DNA and ss-RNA can interact with the TLRs [66].

The response of pDCs to stimulation has been experimentally explored by the use of model ligands for TLR7 and TLR9. In addition to viral ss-RNA, ligands for TLR7 include synthetic compounds of the family of imidazoquinolines [67]. TLR9 on pDCs can be triggered by specific unmethylated CpG-containing motifs common to both bacterial and viral DNA. Such DNA can be mimicked by synthetic oligodeoxynucleotides (ODN) that contain such CpG sequences that stimulate pDCs [68]. Whereas most responses of pDCs have been shown to be TLR-dependent, one study suggested that BM-derived pDCs can also respond to HSV-1 in a TLR9- and MyD88-independent manner [69]. Moreover, recent evidence suggests that cooperative action of TLRs and other surface molecules influences the activation of pDCs. These molecules include the Fc receptors for IgG ($Fc_{\gamma}RIIa$) and for IgE ($Fc_{\varepsilon}RI$), Siglec 5, and the scavenger receptors CD36 and CXCL16 [70].

4.3.2.2. Downstream signaling pathways of TLR7 and TLR9

TLR triggering in pDCs results in the activation of a number of cross-connected intracellular signaling cascades (Figure 5) in a spatiotemporally regulated manner. Signals transmitted through TLRs including TLR7 and TLR9 involve the recruitment of the adaptor molecule MyD88 (myeloid differentiation factor 88) and TRAF6 (tumor necrosis factor receptor-associated factor 6). Both adaptors build a complex together with IRAK1 and IRAK4 (IL-1R-associated kinase 1 and 4) that results in the phosphorylation of the transcription factors IRF7 and IRF5 (interferon regulatory factor 7 and 5). This pathway is essential for transcription of type I interferon genes [71-75]. In comparison to other cells, pDCs constitutively express high levels of IRF7 that enables them to immediate transcription of type I interferons [76].

IFN-α and IFN-β can act in an autocrine and paracrine manner by binding to the IFN-α/β (IFNAR) receptor, which activates the canonical JAK/STAT pathway. Activation of janus kinases JAK1 and JAK2 leads to phorphorylation of the downstream targets STAT1 and STAT2 (signal transducer and activator of transcription 1 and 2). These factors build a heterodimeric transcriptional activator complex, and can also form a heterotrimeric complex

with IRF9 termed ISGF3 (interferon-stimulated gene factor 3). In the nucleus, they bind to ISRE (IFN-stimulated regulatory elements)-containing promoter sequences of a large number of target genes known as IFN-stimulated genes (ISGs). Similarly, transcription of IRF7 induces production of large amounts of type I interferons in a positive feedback loop.

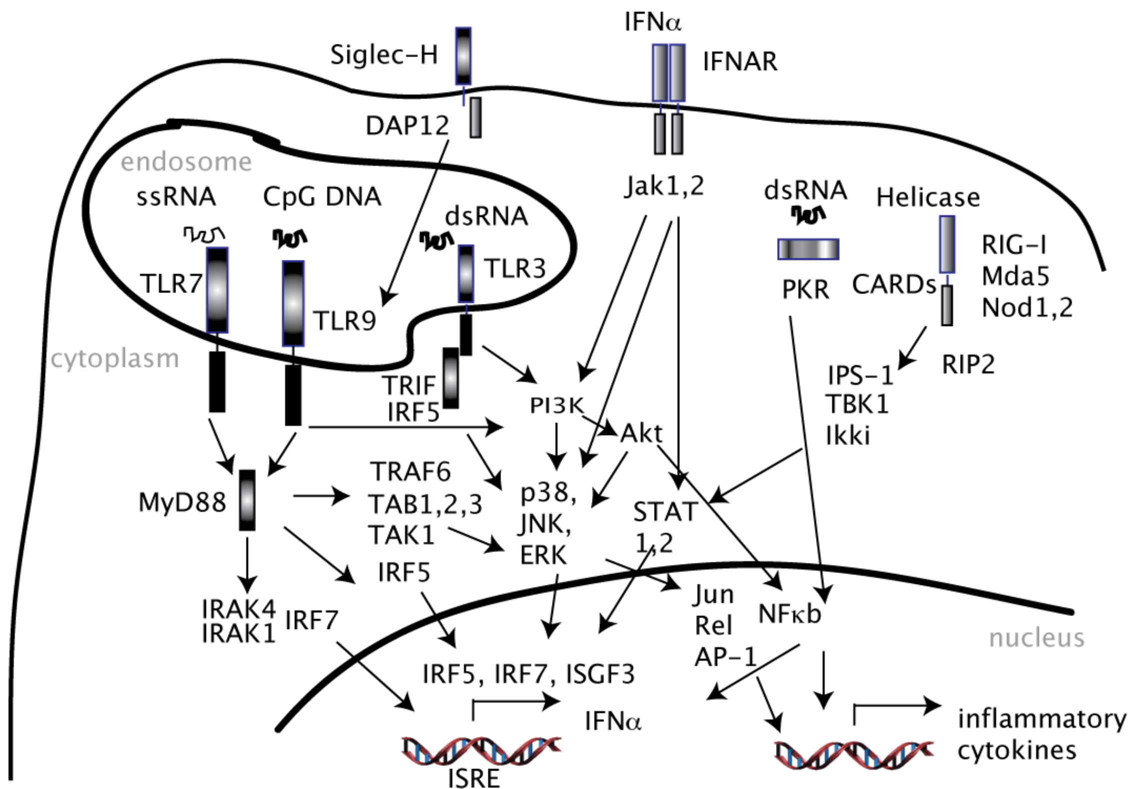

Figure 5: Schematic of Toll-like receptor signal transduction pathways.
(see text for details)

In addition, intracellular signals following TLR7 and TLR9 activation are transduced through the stress kinases of the MAPK- (mitogen-activated protein kinase), ERK (extracellular signal-related kinase)- and JNK (Jun-N-terminal kinase)- family, which lead to I-κB (Inhibitor of NF-κB) degradation and the activation of the transcription factor NF-κB that is essential for the production of pro-inflammatory cytokines and chemokines [77, 78].

In human pDCs it has been shown that p38-MAPK is also involved in CpG-induced IFN-α and chemokine production [75, 78]. The use of knockout mouse strains in several signaling components suggests a complex interaction and possible dissociation between the IRF-pathway for induction of type I interferons, and the NF-κB pathway for transcription of cytokines during differentiation of pDCs [73, 79]. pDCs from mice deficient in NF-κB1 and c-Rel show decreased production of IL-12, whereas IFN-α level are normal after TLR9 signaling induced by CpG ODN [79].

With the recent identification of Siglec-H in pDCs, investigators started to examine possible negative regulators of pDC function. Siglec-H associates with the signaling adaptor DAP12 (DNAX-activating protein of 12 kDa) and was shown to inhibit IFN-α secretion [45, 47, 80, 81]. SOCS (suppressor of cytokine signaling) proteins and other signaling regulators such as src-kinase, phosphoinositide 3-kinases (PI3Ks), Ikkb-kinase and small GTPases [71, 82-86] are also involved in TLR signaling, however their precise roles in pDCs remain to be determined.

4.3.3. Role of activated plasmacytoid dendritic cells in immune responses

pDCs are thought to play a central role in coordinating immune responses (illustrated in Figure 6). With the capacity of pDCs to produce copious amounts of type I interferons, their role on other cell types has been intensively investigated. Type I IFNs have direct antiviral activity as they sensitize infected cells to apoptosis to interfere with viral hijacking of the host cell machinery, and they prevent replication and spread of virus [87]. Interferons can act in a paracrine manner notifying surrounding cells of viral infection, and inducing transcription of a variety of genes with antiviral functions. During viral infections, type I interferons can induce maturation of dendritic cells and ‘license’ APCs for a process termed cross-priming, when $CD8^+$ T cells are primed against exogenous antigens [88, 89]. Type I IFNs can also regulate two major effector cell populations, NK cells and CTLs. Interferon α/β can enhance NK cell cytotoxic activity, and stimulate (bystander) proliferation of certain

T cell subsets [46, 90-92]. In contrast, during systemic infection with lymphocytic choriomeningitis virus (LCMV), IFN-α/β is thought to negatively regulate IFN-γ expression in NK and T cells [93]. Finally, pDC-produced IFN-α can induce expression of TRAIL (TNF-related apoptosis-induced ligand) on monocytes, NK cells and T cells [94]. TRAIL is involved in target-cell killing of virus-infected cells as well as tumor cells. Upon contact with influenza virus, TRAIL is upregulated on pDCs themselves, suggesting that pDCs can contribute directly to elimination of target cells [95].

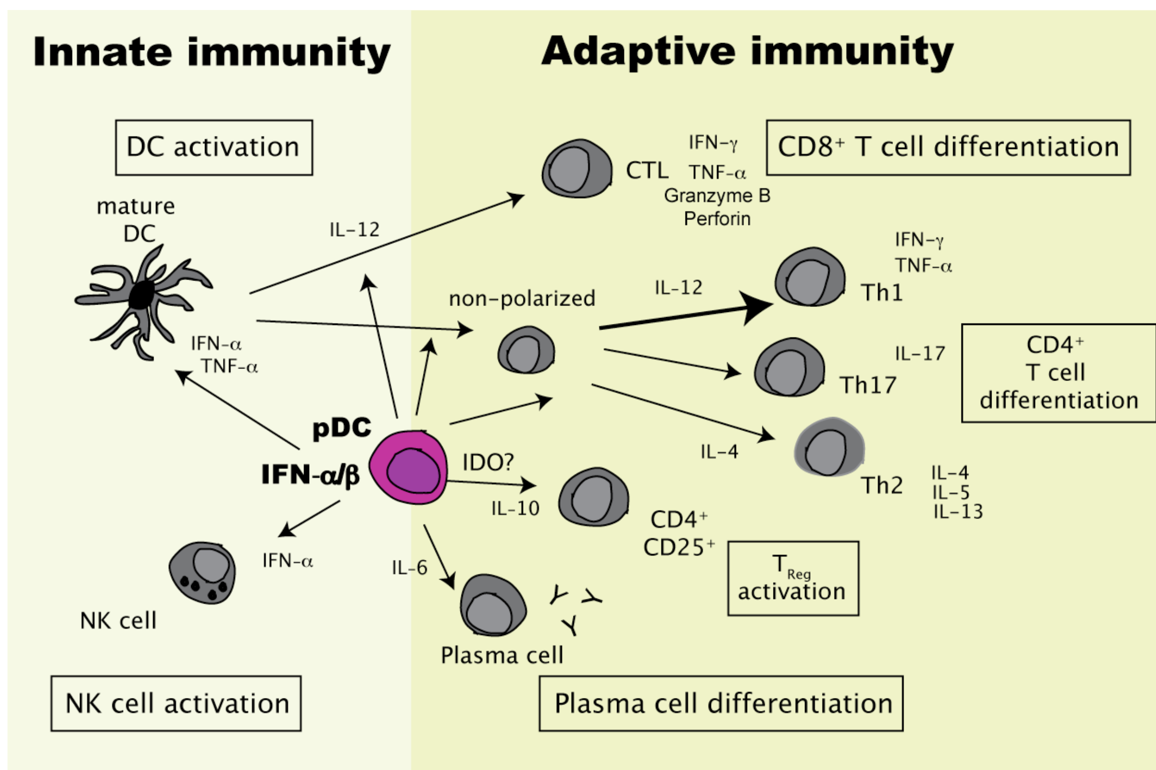

Figure 6: Schematic of pDCs as a central link between innate and adaptive immune responses.

(see text for details)

Whereas type I interferons can promote dendritic cell maturation and induce production of IL-12 necessary for the Th1-type immune response, murine pDCs themselves can produce IL-12 and TNF-α to a variety of TLR ligands and viral stimulation [39, 46, 96]. In addition, type I interferons and IL-6 produced by pDCs can augment B cell differentiation into plasma cells indicating an important role for pDCs in the secretion of antiviral antibodies [97-100]. However, it is unclear whether this process is mediated in a $CD4^+$ T helper dependent or –independent manner, and whether the cytokines released by pDCs may in fact act on follicular dendritic cells that in turn enhance survival of plasma cells through B cell activating factor (BAFF) and proliferation-induced ligand (APRIL) and/or other cytokines such as IL-10 and IL-15.

Finally, pDCs may play a role in antigen presentation and T cell differentiation, either directly or indirectly via IL-12 production and/or other cytokines,. Activated pDCs can upregulate cell surface expression of costimulatory molecules and MHC class II, thus increasing their T cell stimulatory capacity. Several groups have demonstrated that pDCs activated by certain viruses or CpG ODN are able to induce expansion of antigen-specific $CD8^+$ T cells and induce $CD4^+$ T cell polarization into Th1 lineage *in vitro* [96, 101-107]. However, other studies questioned the APC-function of pDCs after influenza virus infection *in vivo*, as $CD8\alpha^+$ DCs, but not pDCs, were shown to be the main antigen presenting cell [108, 109]. It is possible that pDCs cooperate with DCs to induce the differentiation of T cell populations that have been initially expanded by DCs [110]. Such a cooperation was recently suggested for the activation of NK T cells (natural killer T cells) [111, 112].

Under certain conditions, pDCs have also been shown to exhibit tolerogenic potential, release IL-10, and to induce $CD4^+$ $CD25^+$ regulatory T cells [113-117]. Stimulation of pDCs with human immunodeficiency virus (HIV), CTLA4-Immunoglobulin (Ig) or CD200-Ig induced the production of indoleamine 2,3-dioxygenase (IDO), leading to tryptophan depletion and the accumulation of toxic metabolites that have an inhibitory effect on T cell proliferation and survival [118, 119].

In addition to cytokines, activated pDCs can produce a variety of chemokines, which are molecules guiding the migratory behavior of cells in the immune system. Adhesion molecules and chemokines and their receptors determine the trafficking pathways of various cell subsets in an organ-specific manner (described in more detail below). When stimulated with CpG ODN or virus, pDCs secreted CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) [120-124]. Supernatants of influenza-infected human pDCs compared to the supernatants of non-activated pDCs could attract monocytes, NK cells and $CD4^+$ T effector/memory cells, a process that was inhibited by neutralizing antibodies to CCL4 and CXCL10 [120, 123, 124]. It is intriguing to speculate that pDCs support the recruitment of effector T cells to the site of inflammation to combat viral pathogens.

4.4. Migratory properties of plasmacytoid dendritic cells

Due to the complex phenotype and the small numbers of pDCs within organs, investigations of their traveling pathways and localization within tissues have been difficult. Under homeostatic conditions, pDCs are present in variety of organs in low numbers, however, they can accumulate in various organs after viral infections and in autoimmune disorders (see details below). Especially under inflammatory conditions, the migratory dynamics of pDCs remain incompletely understood.

4.4.1. Leukocyte migration paradigm

Trafficking (or homing) of leukocytes from the blood into organs occurs in a highly specific and organized manner. The process is mediated by a cascade of consecutive multistep interactions between the cells in the blood and the endothelial cells of the vessel wall (reviewed in [125]). A schematic representation of these steps is illustrated in Figure 7.

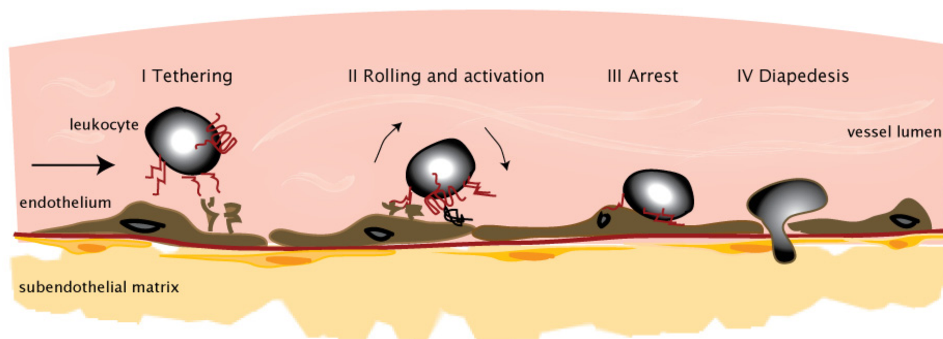

Figure 7: Multistep adhesion cascade of leukocyte extravasation from the blood vessel into the surrounding tissue

To summarize, the initial interaction is mediated by members of the selectin family and α_4 integrins and their ligands (see Appendix, Table XVI and Table XVII) that results in leukocyte tethering and rolling along the vessel wall. These molecular interactions are usually too short-lived to mediate firm arrest of the rolling cell. Chemokines displayed by endothelial cells are necessary for the next step, and bind to $G\alpha_i$-protein-coupled receptors on leukocytes (see Appendix, Table XV). This signal activates integrins to mediate firm arrest. In a final step, leukocytes transmigrate through the blood vessel wall.

4.4.2. Localization and migration of pDCs in the steady-state

pDCs have been identified in lymphoid organs (BM, thymus, LNs and spleen) as well as in lung, liver, skin, gut and peripheral blood [17, 18, 40, 45, 126, 127]. In mice, their frequency and number is very low and variable between strains [18, 40]. In secondary lymphoid organs, pDCs are located in T cell-rich areas. In spleen, pDCs are found preferentially in periateriolar lymphoid sheaths, and some scattered in the marginal zone and the red pulp [128]. While most mDCs enter LNs from peripheral tissues via afferent lymphatics, pDCs enter via the high endothelial venules (HEV) in an L-selectin-dependent manner similar to naïve lymphocytes [40, 129]. In addition to L-selectin, blood-borne pDCs express the P-selectin ligand, PSGL-1 (P-selectin glycolipid-1) [129]. BM-derived as well as

blood-borne pDCs express lymphocyte function-associated antigen, LFA-1, which binds to intercellular adhesion molecules (ICAMs) on the blood vessel endothelium. pDCs also express the integrins $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5), receptors for vascular cell adhesion molecule-1 (VCAM-1) and fibronectin, respectively [129, 130].

In addition to adhesion molecules, immature pDCs express a variety of chemokine receptors such as CXCR3, CCR2, CCR5 and CXCR4 [116, 121, 131]. However, despite the expression of the corresponding receptors, pDCs do not respond efficiently to CXCR3 ligands [132], nor to CCL2 or CCL5 [133], ligands for CCR2 and CCR5. Interestingly, the response of pDCs to CXCR3 ligands may require CXCL12 [121] or vice versa [134]. *In vitro* studies have shown that immature pDCs are attracted by CXCL12 (SDF-1α), the ligand for CXCR4 [130, 133], possibly supporting the migration into lymphoid organs as well as inflamed tissue. In addition, ChemR23, the ligand for chemerin, which is expressed on HEV in secondary lymphoid organs and inflamed skin, was identified on pDCs [135, 136]. Although pDCs are present in a very small number in peripheral organs under homeostatic conditions, it remains unknown which mechanism(s) mediate their entry and/or exit into/from those organs.

4.4.3. PDC trafficking to sites of inflammation

Several studies have shown that blood-borne pDCs accumulate in lymph nodes draining the sites of inflammation [39, 45, 129, 131]. For this process, pDCs require L-selectin for rolling and β_1 and β_2 integrins for firm attachment to the inflamed HEV wall [129]. The accumulation of pDCs in inflamed LNs is inhibited by blocking CXCL9 alone or in combination with anti-E-selectin antibody, suggesting that CCR3 and E-selectin ligands are involved in pDC entry into LNs. It was also shown that after activation, pDCs downregulate the expression of CXCR3 and CXCR4, and upregulate CCR7 [39, 121]. Thus, activated pDCs acquire the ability to migrate in response to the CCR7 ligands CCL21 and CCL19 [121, 128, 133] suggesting that pDCs, similar to mDCs, use CCR7 for homing and localization within secondary lymphoid organs. A possible contribution of other

inflammatory mediators and "unconventional" chemoattractants, such as adenosine and the complement components, C3a and C5a, remain speculative [137, 138]. Even though pDCs have been found in increased numbers in lungs after infection with respiratory syncytial virus (RSV) and inflamed tissue such as synovial fluid from patients with rheumatoid arthritis (RA) or skin lesions of patients with systemic lupus erythematosus (SLE) (see below), it is unknown by which mechanisms pDCs infiltrate such tissues.

4.5. The role of plasmacytoid dendritic cells in pathology

4.5.1. Plasmacytoid dendritic cells in viral infections

Recent evidence suggests that the contribution of pDCs to the antiviral immune response reaches beyond the mere production of type I interferons. As mentioned before, pDCs have been shown to produce a variety of chemokines [120, 123] that may contribute to the recruitment of NK cells and effector T cells to the site of infection. In addition, pDCs may exhibit direct cytotoxic effector functions through the upregulation of TRAIL on their own surface [95]. Several clinical studies suggest that pDCs appear to be decreased in number or defective in function in certain chronic viral infections such as hepatitis infections and acquired immunodeficiency syndrome (AIDS). In patients with chronic hepatitis caused by hepatitis B and hepatitis C virus, peripheral blood pDCs were significantly reduced in numbers and in their capability to produce IFN-α, which may favor viral persistence [139, 140]. Due to the expression of CD4, CCR5 and CXCR4, which are molecules that allows human immunodeficiency virus (HIV) entry into cells, pDCs are targets for this virus, and have been shown to transfer HIV to $CD4^+$ T cells [141-143]. Moreover, the loss of pDCs in AIDS-patients correlated with a high viral load [144]. A recent study has linked HIV-induced expression of the immunosuppressive enzyme IDO in pDCs to the possible impairment in the $CD4^+$ T cell response [119]. It was found that the elderly, who are more susceptible to

mortality caused by certain viral infections, have a deficit in numbers of circulating pDCs [145, 146].

4.5.2. Plasmacytoid dendritic cells in autoimmune disorders and other diseases

Besides microbial recognition, endogenous ligands, such as DNA- and RNA-containing immune complexes of self-antigens have recently been implicated to activate pDCs. Moreover, elevated level of type I interferons have been associated with a variety of autoimmune disorders such as SLE, RA, Sjogren's syndrome, psoriasis and diabetes [147, 148, 149, 150]. It has long been known that patients with SLE have increased serum levels of type I interferons [151], which were found to activate mDCs to trigger T-cell mediated autoimmunity [152]. pDCs of patients with SLE accumulate in skin lesions [153]. The mechanism of their activation possibly involves chromatin-containing immune complexes such as autoantibodies and nucleic acid products from apoptotic/necrotic cells that are internalized by pDCs after their binding to Fc receptors. After their endocytosis they reach the endosomal compartment and activate TLR7 and/or TLR9, which results in type I interferon production [154-158]. Interestingly, new findings suggest that TLR7 and TLR9 may have opposing effects on regulating the activation status of pDCs, and may therefore differentially regulate autoantibody production in SLE [159].

Type I interferons and pDCs may also play a role in tumor immunology (for review see [160]). Depletion of pDC-derived IFN-α abrogated the expression of TRAIL on monocytes/macrophages and therefore negatively impacted their anti-tumor activity [94]. PDCs in tumor-draining lymph nodes were shown to express IDO, which may contribute to the unresponsiveness of T cells against tumors [118]. Not only in tumor immunology, however, have pDCs been associated with negative immune regulatory functions. Tolerance induction by pDCs has been proposed in two recent studies of transplanted cardiac allografts [113], hematopoietic stem cells and skin grafts [161]. Furthermore, in a murine model of

asthma, pDCs prevented excessive inflammatory responses; thus, the absence of pDCs achieved by depletion increased the sensitization to an inhaled antigen resulting in allergic reactions [17, 24].

In summary, emerging evidence suggests multiple roles of pDCs during physiologic and pathologic reactions, which warrant a detailed characterization of their activation, migration and immunologic functions in health and disease.