9. General Discussion

In the crucial discrimination between self and non-self or non-infectious and harmful, the innate immune system has to recognize physiological from pathological signals. It does so by a number of evolutionary conserved receptors that allow for the recognition of pathogen-associated patterns (PAMPs). The family of Toll-like receptors (TLRs) plays an essential role in recognizing a wide range of PAMPS (see Table I). Among the innate immune cells expressing these receptors are neutrophils, macrophages, DCs and NK cells. Stimulation of TLRs triggers signaling pathways that result in the induction of antimicrobial genes, pro-inflammatory cytokines and chemokines. The activated cells transform into effectors ready to battle invading pathogens, and to notify other cells of the source of infection. DCs also instruct antigen-specific T and B cells, part of the adaptive immune response, to fight the pathogens. This leads to clonal expansion and the differentiation into effector T cells and antibody-producing B cells. The quality and quantity of the response is determined by the nature and the amount of the pathogen. It is still incompletely understood how innate immune cells can communicate such complex information to adaptive immune cells leading either to eradication of pathogens, or, in the case of their failure, to persistent infections. Moreover, dysregulations of antimicrobial immunity may be linked to other important diseases, in particular asthma and autoimmune disorders. Therefore, understanding how innate immune responses are regulated is important for unraveling the pathogenesis of infectious diseases, and may lead to novel therapeutic strategies in situations where infections cannot be cleared.

Plasmacytoid dendritic cells (pDCs) were recently identified as a link between innate and adaptive immune processes. Key mediators in this communication are type I interferons and other cytokines and chemokines that pDCs secrete at high levels following activation. While pDCs have been considered important contributors of antiviral cytokines during infections, the precise role of pDCs in infections *in vivo* has not been thoroughly studied. The work presented in this thesis was undertaken to investigate the functional significance of pDCs in the response to influenza virus infection. In chapter 1, we described the distinct

maturation and differentiation characteristics of pDCs following stimulation with influenza virus as compared to CpG containing DNA oligonucleotides. In chapter 2, the molecular mechanism of pDC migration to sites of inflammation was studied. Finally, the results presented in chapter 3 suggest that pDCs are involved in early T cell recruitment to the influenza virus-infected lungs, but are dispensable for viral clearance. In the following sections, the major findings of each chapter will be summarized and discussed in the context of the current literature and future directions.

9.1. Differentiation of activated pDCs – a model for two distinctly polarized subsets

It has previously been hypothesized that activation of immature pDCs can result in differentiation into type I interferon (IFN) producing cells, which can then further develop antigen presenting capacities similar to conventional dendritic cells [107]. However, the molecular processes underlying the transition of immature pDCs into differentiated pDC subsets are not well understood. Moreover, it has been unclear whether the differentiation of IFN-producing cells can be dissociated from the pathway of differentiation into "dendritic" pDCs [176]. The study presented in chapter 1 deciphered in detail the differences between pDCs activated by two different TLR7 and TLR9 ligands, PR8 influenza virus and CpG 1826 oligonucleotides. For this work, a recently described transgenic mouse model was used, DPE^{GFP}, in which pDCs express high levels of GFP. This allowed us to obtain a pure pDC population without contaminating mDCs and B cells. To the best of our knowledge, such pure pDCs have not been isolated *ex vivo* in the past. Thus, we were able to conduct functional experiments that determined the response of pDCs to prototypic TLR ligands without the danger of confounding cell contaminations.

We employed global gene transcription profiling in combination with phenotypic and functional analyses of pDCs activated by influenza PR8 virus and CpG 1826. This stimulation resulted in the induction of a large number of antiviral and interferon-induced

genes shared by both stimuli, indicating the existence of a common response regardless of the nature of the pathogen. However, similar to what has been observed for mDCs and their plasticity in response to various pathogens, such as bacteria, fungi and viruses [195], pDCs exhibit, at least in part, a pathogen-specific response pattern as evidenced by distinct cytokine and chemokine profiles at the mRNA as well as at the protein level. This may result in the dissociation of pDC functions following TLR-induced maturation in a pathogen-dependent manner.

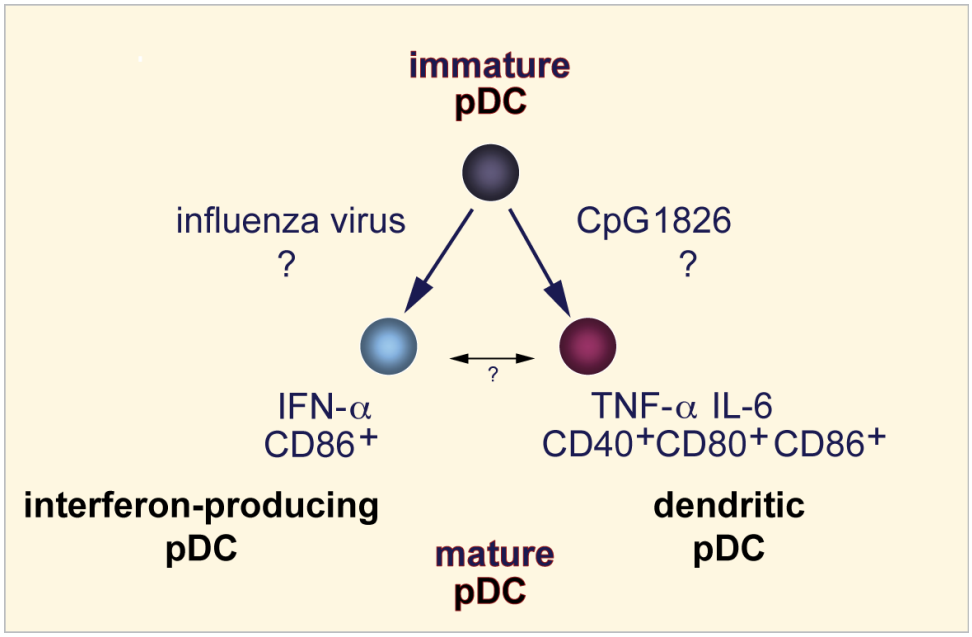

Figure 45: Proposed model of pDC differentiation following activation.

Immature pDCs can differentiate into two phenotypically and functionally distinct populations. After activation with influenza virus, pDCs produce high amounts of IFN-α, but acquire only a partially mature phenotype compared to pDCs stimulated with CpG 1826, which upregulate CD40 and CD80 in addition to CD86 and produce pro-inflammatory cytokines.

Based on our findings, we propose a model that suggests that murine pDCs can be differentially polarized into a population defined by the intermediate expression of costimulatory molecules and the secretion of high amounts of IFN-α (after PR8 virus

stimulation (see Figure 45)). A second differentiation state induced by CpG 1826 is characterized by the production of high levels of pro-inflammatory cytokines and chemokines, increased expression of costimulatory molecules and a DC-like morphology. The generation of gene expression signatures for interferon-producing pDCs and dendritic pDCs, as performed here, opened opportunities to study the involvement of subset-specific genes in their differentiation.

Despite these advances, several important questions remain to be answered. First, how do pDCs discriminate encoded signals from TLR7 and TLR9? Phylogenetically, TLR7 and TLR9 are highly homologous and participate both in the recognition of nucleic acid-like microbial components. Signals of these receptors are transmitted through the common adaptor molecule MyD88, suggesting at least in part a common response. However, divergence of intracellular signaling pathways are likely to occur downstream of MyD88 (see Figure 5). We demonstrated that important signal transducers, such as p38 kinase, ERK2 and a number of transcription factors are differentially activated. Moreover, after viral entry or CpG-uptake, different mechanisms of endocytic trafficking may impact the differentiation process and induce separate signaling cascades inside the cell. Recent studies have shown that distinct intracellular localization and retention of CpG-subtypes within the cell correlated with levels of IFN-α production [198]. For instance, CpG-A oligodinucleotides are potent inducers of IFN-α and are retained in the early endosomes, whereas B-type CpG, which are weak inducers of IFN-α, are distributed in lysosomes [199]. As both types of CpG bind to TLR9, the results suggest that the nature of the antigen dictates such distinct responses. Thus, it is likely that CpG A induces a comparable response to what we have observed for pDCs stimulated with PR8 virus. In addition, Gursel et al. identified the scavenger receptor CXCL16 as a modulator in the response of pDCs to different CpG-types [70]. It is therefore conceivable that heterophilic interactions between TLRs and other innate receptors may contribute to the differential response of pDCs to various stimuli. Identification and characterization of such molecules and their adaptors will help to illuminate the processes following activation of pDCs.

It is clear from other viruses such as HIV that entry into cells requires co-receptors, such as CCR5 or CXCR4 in addition to CD4, or integrins, glycolipids and proteoglycans for other viruses [221]. It is unclear whether influenza viruses use receptor-dependent mechanisms in addition to receptor-independent endocytotic processes in pDCs. Virus uptake could also be involved in initiating intracellular signaling cascades prior to binding to TLR7. The uptake of influenza viruses into pDCs has not yet been examined in detail and it is also unknown how they are processed inside pDCs. A number of pathogens have developed strategies to evade the induction of pDC-derived type interferon. Strains of RSV and measles virus were recently identified to escape recognition by TLRs, and to block type I interferon production by pDCs [222, 223]. Recent studies reported that the influenza A virus NS1 protein is an important virulence factor suppressing interferon production, attenuating maturation of mDCs [212, 224, 225] and suppressing antiviral responses of lung epithelial cells [226]. Whether this is also true for pDCs remains to be shown. A better understanding of how type I interferons and other cytokines are induced and regulated in pDCs may lead to new therapeutic strategies to interfere with interferon-inhibition by viruses. Furthermore, deciphering the steps of viral entry into cells, an obligatory process prior to viral replication, could lead to the development of more effective antiviral vaccines.

Finally, what may be the relationship between and the functional significance of distinct pDC subpopulations in disease *in vivo*? pDCs may be able to undergo conversion from high interferon-producers to antigen presenting ($CD11c^+$ $CD11b^+$) mDCs, as has been suggested by a study using bone marrow pDCs activated with LCMV [107]. However, under neither of the conditions used in this study (influenza PR8 virus and CpG 1826 ODN) did we observe expression of CD11b on pDCs. Our findings are in line with others that have suggested that pDCs from the spleen are more differentiated than the precursors from the bone marrow, which may not be uniform and/or harbor distinct cell subsets that can give rise to different cells [227]. It appears to be clear that pDCs are a distinct lineage from mDCs, but their exact relationship during development from hematopoietic stem cells is still controversial. In the future, it will be important to study whether PR8-activated pDCs can convert to dendritic pDCs, or whether both subsets represent the final differentiation states. It is likely that

autocrine IFN-α is necessary to maintain the phenotype of PR8-activated pDCs (see Figure 16). In addition, it has been shown that human pDCs activated by HSV become refractory to IFN-α production after maturation with IL-3 and CD40L [38] suggesting that dendritic pDCs may not be able to convert to interferon-producing pDCs. Thus, the functional difference between the two activation states appears to be programmed early after activation.

However, with respect to temporal changes in the course of viral infections, it is conceivable that the functions of pDCs may change over time. We can envision a scenario similar to the model of "exhausted dendritic cells" [228], in which mature mDCs have a certain time window after their activation before they become unresponsive and induce T cell activation insufficiently. Thus, it is possible that type I interferon or any pro-inflammatory cytokine production is only a transient feature of activated pDCs, while later on, they act as negative immune regulators. We observed that CpG-activated pDCs produced IL-10, which has immunosuppressive functions, and may contribute to limiting inflammatory responses. A number of negative regulator proteins, such as members of the SOCS family, were also induced upon activation of pDCs. We hypothesize that the events occurring during activation of pDCs, which promote inflammation may also be programmed for a coordinated "self-limited resolution", i.e. the acquisition of anti-inflammatory functions as described above. Others have shown that pDCs stimulated with HIV produce IDO or induce regulatory T cells upon activation with CpG [115, 119], which in turn could limit inflammatory responses. Recognition of endogenous (self) nucleic acids in form of immune complexes during inflammation, tissue injury or increased cell death can trigger activation of pDCs, which breaks tolerance and results in autoimmunity. Thus, a better understanding of negative immune regulators produced by pDCs or exogenous factors negatively regulating their functions may increase our knowledge of how to potentially modulate the pathogenesis of viral and autoimmune diseases. Whereas our studies were mainly focused on antiviral aspects of pDC functions, further studies will be needed to decipher how pDCs respond in detail to other pathogens. As CpG sequences are also prevalent in the DNA of bacteria and the genome of DNA-viruses, it is likely that studies of pDC responses to such pathogens may uncover additional functions.

In summary, pDCs appear to exhibit high levels of plasticity in their functions depending on the receiving activation signal. It is likely that this flexibility reflects the necessity for pDCs to differentiate into subsets with the capacities most suited to fight against an invading pathogen.

9.2. Migratory properties of pDCs – cues to their function?

The paradigm of DC migration suggests a functional link between migration and differentiation. DC precursors exit from the bone marrow to the blood, from where they migrate into organs. Within tissues, immature DCs act as sentinels for danger signals and survey the microenvironment for invading pathogens. Once DCs recognize PAMPS from invading microbes, they take up antigen and mature. During this process, they downregulate their phagocytosis-machinery, process the collected antigen and upregulate costimulatory molecules in addition to MHC molecules for antigen presentation. A switch in expression of trafficking molecules, in particular the upregulation of CCR7, initiates their migration from peripheral tissues via afferent lymphatics into draining lymph nodes where they activate naïve T cells. This pathway has been well described for migrating Langerhans cells from the skin and lung mDCs [20, 21]. However, DCs can be divided in several subpopulations, which complicates this simplified paradigm. Thus, it is conceivable that pDCs which do not have classical antigen presenting functions, but rather regulate the local environment by secreting cytokines and chemokine (see chapter 1 and chapter 3), also differ in their migratory routes from DCs. Since we observed that pDCs are recruited to the inflamed lungs following influenza infections, and relatively little is known about the trafficking pathways of pDCs *in vivo*, we set out to study their homing behavior.

We showed that blood-borne immature pDCs expressed a broad panel of adhesion molecules including P- and E-selectin ligands, CD44, CD11a (α_L), CD18 (β_2), CCR2, CCR5, CXCR3 and CXCR4 (Table XIII). In contrast to mDCs, however, pDCs express high levels of L-selectin (Figure 26) and were found to enter PLN from the blood (Figure 28). Although

L-selectin mediates rolling on HEV as shown by Diacovo et al. [129], immature pDCs do not express CCR7, which, at least for T cells, initiates firm adherence. It is possible that pDC-expressed CXCR4 binding to SDF-1α and/or the chemerin receptor binding its ligand expressed on HEV could support this process [134, 135]. Epifluorescent intravital microcopy (IVM) [229] in combination with our GFP-transgenic mice will allow for the investigation of specific adhesive interactions of endogenous pDCs in the vasculature of LNs. While Diacovo et al. found only limited transmigration of pDCs into lymph nodes under homeostatic conditions, he used IVM to study the adhesive mechanisms of pDCs on inflamed HEV [129]. However, in this study BM-derived pDCs were expanded *in vitro* with Flt-3L, purified with magnetic beads and then fluorescently labeled for the experiments. Such a manipulation may carry the risk of altering the migratory behavior. To the best of our knowledge, our GFP-transgenic mice represent the only tool available to visualize endogenous pDCs *in vivo* and will be useful to study pDC migration in a variety of models established for IVM including skin, BM, liver, eye, cremaster muscle, intestine and pancreas in addition to lymph node preparations (for review see [230]).

In addition to addressing the question of how pDCs enter lymph nodes, we can ask why a small number of immature pDCs may home from the bloodstream through HEV into secondary lymphoid organs and localize there in T cell areas. We can speculate that immature pDCs in the blood may not only be part of an immunosurveillance program of the entire body, but may also play a role in maintenance of tolerance. In line with this idea, it has recently been shown that pDCs in an allograft model can take up alloantigens from the graft by phagocytosis and subsequently migrate via the blood stream into lymphoid organs, where they induce regulatory T cells [113]. While this model may not reflect the conditions in the steady-state, another study could demonstrate that DC homing from the blood stream into the thymus carry self-antigens and induce deletion of antigen-specific thymocytes [231]. Although we did not investigate homing into the thymus, we found that pDCs have high mRNA levels of CCR9, which mediates chemotaxis in response to CCL25/thymus-expressed chemokine, and that pDCs are present in the human thymus [127], where they may be

involved with negative selection of autoreactive thymocytes. Along with other publications which support tolerizing functions of pDCs during immune responses [17, 118], our findings of the discrete homing behavior of pDCs into secondary lymphoid organs may be important and warrant further molecular characterization.

In addition to our observation that pDCs are able to enter most organs from the blood stream (Figure 28), we demonstrated that pDCs home well to sites of inflammation. In the peritonitis model, pDCs harvested from the peritoneal cavity expressed low levels of costimulatory molecules, suggesting that they are recruited as immature cells and become activated locally when they encounter specific ligands such as CpG or influenza virus (Figure 30). MDCs, in contrast, exhibited a more mature phenotype. Importantly, the expression of homing molecules of recruited pDCs differed strongly from the profile of mDCs, suggesting that distinct recruitment mechanisms may regulate influx of pDCs versus mDCs to sites of inflammation. We deciphered the importance of L-selectin in combination with P- and E-selectin, required for pDC recruitment to PEL (Figure 31). However, the peritonitis model may differ in the inflammatory conditions from influenza infection in the lungs. While we found increasing numbers of pDCs in lungs and airways during this infection, the precise molecular mechanism of pDC recruitment into these compartments remains to be studied. Others have found a number of chemokines with different expression kinetics in lungs after infection with PR8 virus infection [211]. Among those were CXCL10 and CCL5, ligands for the chemokine receptors CXCR3 and CCR5 expressed by pDCs, respectively. pDCs also express CD44, LFA-1 and α4 integrins, which can bind to their counterligands hyaluronic acid, ICAM-1/2 and VCAM-1 on lung endothelial cells. Using knockout mice deficient in candidate molecules, we will determine in the future whether pDC homing into influenza virus-infected lungs in those animals is impaired.

Our studies revealed another important aspect of pDC migration and how their homing may be regulated not only by exogenous factors but, for example, by the regulation of expression levels of homing molecules on their surface. Thus, we have found high L-selectin expression on circulating, but low levels on splenic and CpG-activated pDCs (Figure 26).

Our data suggest that tissue-resident and activated pDCs have downregulated levels of this molecule. While the significance of this modulation is unclear, the regulation of L-Selectin expression on the surface of neutrophils and lymphocytes has been studied in detail (for review, see [232]). The ectodomain of L-selectin is proteolytically cleaved in a process termed "shedding" and results in diminished leukocyte-endothelial cell interactions. One of the enzymes responsible for this cleavage is the TNF-α converting enzyme (TACE, ADAM17). Corresponding to the lower L-selectin expression after pDC activation with CpG 1826, we found a 4-fold increase in mRNA levels of TACE in our microarray experiment (data not shown). Our results suggest an additional mechanism, by which migratory pathways of pDCs may be regulated. As shown in Figure 31, inhibition of L-selectin interferes with homing of pDCs into the inflamed peritoneal cavity. Using chimeric mice that contain cells with L-selectin resistant to shedding [233], it will be interesting to study whether pDC homing is altered in these mice. We can envision two possible results of these experiments. As L-selectin has been shown to mediate rolling and adherence of pDCs on HEV in LNs [129], it is conceivable that blocking the downregulation of L-selectin could result in decreased velocity due to prolonged interactions with L-selectin ligands, which can lead to higher chance of cell adherence and subsequent accumulation. In line with this hypothesis, studies of neutrophil migration have reported similar results [234]. However, when pDCs extravasate from the blood into organs, L-selectin shedding may be necessary for transendothelial migration [235]. Inhibition of L-selectin shedding may therefore prevent influx of pDCs into organs. While it has been shown that downregulation of L-selectin prevents activated T cells from re-entry into lymph nodes [236], it remains to be determined, how the modulation of L-selectin regulates trafficking of activated pDCs. A better understanding of the key molecules guiding migration of pDCs into sites of inflammation will be important in order to interfere with their selective homing to target organs, in particular in autoimmune disorders.

Finally, we know little about the migratory pathways of pDCs within the tissues after their extravasation. While we have found that chemotactic mediators are possibly involved in

the migration of pDCs to sites of inflammation (Figure 29), chemokines are not only displayed on the endothelium, but are also thought to determine the cell's localization within organs. The prevailing hypothesis has been that migrating cells sense a gradient of chemokines, which provides guidance towards the source of their release. Recent studies using intravital microscopy techniques (IVM) combined with two-photon excitation have shed light on cell motility and the dynamics of immune cell interactions deep within tissues (reviewed in [237, 238]). Two-photon (2P) microscopy generates sectional scans through a tissue, which can be reconstructed into three dimensional (3D) time-lapse movies to visualize the dynamics of immune cell migration, for example, DC-T cell- as well as T cell-tumor cell interactions [178, 237, 238]. Using such an approach in combination with our GFP-tagged pDCs, it will be interesting to investigate how pDCs localize and travel within tissues and what cell-cell contact they establish in the steady-state or during inflammation. In preliminary experiments, we set out to study whether pDCs interact with T cells. As $DPE^{GFP}xRag\text{-}1^{-/-}$ mice may have a disturbed microenvironment in lymph nodes due to the lack of B and T cells, we generated bone marrow chimeras that result in WT mice harboring GFP^{hi} pDCs. We then transferred $DsRED^{+}$ T cells into these mice and imaged them together with GFP^{hi} pDCs in an excised PLN (Figure 46). Figure 46 represents a snapshot from our observation. In the absence of any antigen it appears that pDCs and T cells do not co-localize, which has been described for mDCs as well [239].

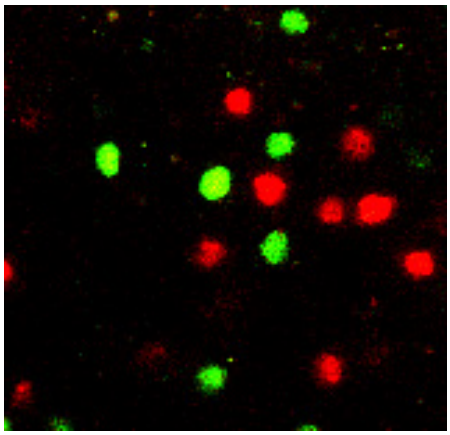

Figure 46: Two-Photon microscopy image of GFP^{+} pDCs and $DsRed^{+}$ T cells.

2P-imaging was performed on an excised lymph node from a chimeric wild-type mouse harboring GFP^{+} pDCs, which had received intravenously $10x10^{6}$ $DsRED^{+}$ cells from a DPE^{DsRED} mouse. The GFP^{+} chimera was generated by irradiation of a WT mouse (900 Rad) and subsequent transfer of $2x10^{6}$ BM cells from a $DPE^{GFP}xRAG\text{-}1^{-/-}$ mouse mixed with $2x10^{5}$ WT BM cells. (Imaging was performed with help from Dr. Paulus Mrass)

While we hypothesize that pDCs do not play a direct role in antigen presentation in influenza virus infection (see chapter 3), a study in a model of cutaneous HSV-1-infection showed that depletion of pDCs downregulated the antigen presenting capacities of mDCs to activate $CD8^+$ T cells [174]. To investigate in detail the immune modulatory functions of pDCs on mDCs during antigen presentation, we plan for the future to transfer YFP-expressing $CD11c^+$ cells [240] together with T cells from OT-1x$DsRED^+$ mice into GFP^+ chimeric mice. After subcutaneous immunization with HSV-1-OVA [241], we hypothesize that GFP^{hi} pDCs influence YFP^+ mDCs during the priming process of $DsRED^+$ T cells in the draining lymph nodes. We will co-visualize the mDC-T cell interactions and test the hypothesis that inhibition of pDC migration into the lymph nodes or the use of pDC-deficient $Ikaros^{L/L}$ mice (see chapter 3) may result in decreased contact times between mDCs and antigen-specific T cells. The absence of pDCs may also inhibit the stable formation of the immunological synapse during priming of T cells. These experiments will further our understanding of how pDCs regulate antigen presentation by mDCs in certain viral infections.

In conclusion, knowledge of pDC trafficking not only from and to certain organs but also within the tissue microenvironments, and the understanding of the underlying molecular determinants, may elucidate the functions of pDCs in the context of a disease. The results of these studies may be useful for finding therapeutic targets for the treatment of diseases by: 1) interfering with their selective homing to target organs, in particular in autoimmune disorders to inhibit chronic inflammation and 2) enhancing the migration of pDCs into organs to promote antiviral immunity or prevent asthmatic symptoms.

9.3. The role of pDCs in viral infections

Respiratory viral infections, such as those caused by influenza virus remain a common threat for the population of industrial and developing countries alike, especially for the elderly and young children. For the control of an infection, the body requires both the innate

and adaptive immune system. As a key link between both systems, pDCs are thought to orchestrate the immune response against viral infections either directly or indirectly. Thus, pDCs have been shown to contribute to the differentiation of plasma B cells, augment the activity of NK cells, attract T and NK cells during viral infections and induce regulatory T cells [92, 96, 97, 113-115, 120, 123, 175]. Evidence for a role of pDCs in viral infections *in vivo* has come from a number of viral models, such as RSV, HSV, vesicular stomatitis virus (VSV), New Castle disease virus (NDV), LCMV and MCMV infections [46, 89, 96, 172-174, 242]. To date, there is only circumstantial evidence that pDCs participate in the anti-influenza immune response. *In vitro* assays have shown that pDCs, upon activation with influenza virus, are able to produce high levels of the antiviral cytokine IFN-α (3-10 pg/cell within 24 h, which is 100-1000 times more than any other blood cell type can produce) [43]. The production of IFN-α did not require viral replication (Figure 15) [243]. In addition to IFN-α, influenza-activated pDCs produced various chemokines that were shown to attract T cells and NK cells in *in vitro* chemotaxis assays (Figure 20) [123]. Moreover, Cella et al. demonstrated that pDCs stimulated *in vitro* with influenza virus can induce $CD4^+$ T cell differentiation. However, the role of pDCs in viral clearance and especially their contribution to T cell responses have not been studied in influenza virus infections *in vivo*. This is partially due to the complex phenotype of pDCs that impedes their unequivocal identification during inflammation. Furthermore, while a mouse model exist to allow *in vivo* ablation of all $CD11c^+$ DCs [244], an animal model with specific deficiency of pDCs but not other DC subsets was not available until to now.

With the use of the recently described mouse strain, $Ikaros^{L/L}$, we were able to address these outstanding questions. Following infection with PR8 virus, we demonstrated that these mice had neither significantly increased morbidity nor a defect in viral clearance. In addition to the lack of IFN-α production in response to MCMV infection *in vivo* [49], we demonstrated that $Ikaros^{L/L}$ mice were unable to produce IFN-α after activation with influenza virus *in vitro*. Several publications using different viral systems have led to confusion about the dependence on pDC-secreted IFN-α for antiviral immunity. We showed previously that the autocrine feedback loop was necessary to sustain pDC maturation and type I IFN

secretion upon *in vitro* activation with influenza virus (Figure 16), while it was demonstrated in a model of VSV-infection that pDCs were able to produce high levels of IFN-α independent of the type I interferon feedback signaling [242].

In another viral model of MCMV infection, but not after challenge with LCMV, pDCs have been shown to be the major producers of IFN-α [188]. Despite a defect of IFN-α production, pDC-depleted MCMV-infected animals were found to have increased levels of IL-12 and IFN-γ in the serum, which was interpreted as a compensatory mechanism from other cells [46]. While the consequences of these shifted cytokine profiles were not investigated in this study, Dalod et al. determined that the cross-regulation of type I IFNs and IL-12 production in response to MCMV was inversely correlated by showing that IL-12 produced by conventional DCs could be limited by pDC-secreted IFN-α/β. Using IFNAR-knockout mice, Honda et al. demonstrated that the lack of autocrine type I IFN inhibited DC maturation and production of pro-inflammatory cytokines upon infection with NDV [89]. Interestingly, IFNAR-knockout mice infected with influenza virus were shown to have relatively normal immune responses, suggesting that pDC-derived type I interferon (and possibly IFN-α/β produced by other cells) is not necessary for clearance of this particular virus and resolution of infection [216]. Our data from pDC-deficient Ikaros$^{L/L}$ mice support this hypothesis as these mice showed signs of normal morbidity and cleared influenza virus similarly when compared to wild-type mice (Figure 37).

Using HSV as the infectious agents, T cell priming was shown to be ablated when pDCs were depleted [174]. However, while others reported that TLR9 and MyD88 are necessary for pDC-responsiveness to HSV, TLR9$^{-/-}$ and MyD88$^{-/-}$ mice were capable of controlling corneal HSV infection *in vivo* [61]. Based on these findings it was unclear, what contributions pDCs make to the activation of T cells during influenza virus infection *in vivo*. We demonstrated that pDC-deficient Ikaros$^{L/L}$ mice had a delay in T and NK cell recruitment into the airways early following infection, which was shown to be pDC-dependent (Figure 41 and data not shown). These data indicate that pDCs are major contributors to chemokine production and to the recruitment of effector cells into the airways. The absence of pDCs appeared to have no negative consequences for viral clearance and resolution of the infection.

Ikaros$^{L/L}$ mice were also shown to not have any profoundly altered B and T cell responses compared to WT mice (Figure 37, Figure 42-44). In addition, conventional CD11c^{hi} DCs appeared to mature normally in response to influenza virus *in vitro* (Figure 36) and *in vivo* (data not shown).

These results suggest that pDCs are either dispensable in this viral model and/or that other cells can compensate for the lack of their functions. As Ikaros$^{L/L}$ mice contain pDC precursors in the bone marrow, we assessed whether these cells can become mature B220^{+} PDCA^{+} CD11c^{int} pDCs during infection. However no mature pDCs could be identified in lungs, LNs and spleens in these mice (Figure 39 and Figure 40). In addition to the use of Ikaros$^{L/L}$ mice, it remains to be shown whether depletion of pDCs *in vivo* prior to infection provides similar results, with the caveat that other cell subsets could potentially be decreased in the process. In contrast to published data [18], we tested the efficacy of the 120G8 antibody to deplete pDCs prior to infection, but we achieved only an incomplete reduction (30 - 40%) of pDCs, which was insufficient for our experiments (data not shown). The Ly6C/G (Gr-1) antibody used by others to deplete pDCs can potentially also decrease neutrophils and was therefore not considered in our studies [188].

Our experiments delineate the early functions of pDCs following influenza virus infection which involve the recruitment of T cells into the airways. Further experiments will be needed to address the role of lung pDCs at later stages after resolution of infection. We showed a massive increase of pDC numbers in mediastinal lymph nodes and lungs during influenza virus infections, which remained elevated long past the clearance of virus. We speculate that pDCs may regulate and suppress other effector cell functions in the late stage after infection, or they may induce regulatory T cells that help to prevent excessive tissue destruction. Recent data indicate that pDCs play an important role in sensitization of mice to inhalation of harmless antigen and suggest a regulatory role of lung pDCs in limiting airway hyperresponsiveness [17]. As some respiratory viral infections have been associated with subsequent allergic occurrence or exacerbation of airway diseases [245], it remains to be

shown how the increase in lung pDCs in response to viral and bacterial antigens impacts the prevalence for allergy and other chronic lung infections.

In summary, while different viral systems, *in vitro* studies and depletion strategies have led to many conclusions about the requirements of pDCs for antiviral immunity, our data suggest that pDCs seem to be dispensable for viral clearance and resolution of influenza virus PR8 infection.

9.4. Conclusions and future outlook

In the future, the model of an infection localized to the respiratory tract studied here will be expanded to investigate the role of pDCs in other viral or bacterial infections, and also during systemic infections. It is not uncommon that viral infections are accompanied by opportunistic bacterial infections. Therefore, pDC functions may become important during a mixed viral and bacterial lung infection. Interestingly, studies have shown that respiratory viruses are a major cause of asthma and chronic obstructive pulmonary disease (COPD) exacerbations. As pDCs have been shown to play an important role in asthma and maintenance of airway tolerance, we hypothesize that they are important contributors to balancing the inflammatory cytokine and chemokine milieu in the lungs.

As the immune functions of pDCs are coupled to their migratory pathways to tissues, we require a better understanding of the molecular cues that mediate their trafficking, especially into sites of inflammation. Clinical trials of a monoclonal antibody against α_4 integrins, nataluzimab, have shown their efficacy in the treatment against multiple sclerosis probably by inhibiting influx of effector cells through interference of $\alpha_4\beta_1$ to its ligand VCAM-1 (reviewed in [246]). While the effect of this drug on pDCs has not been studied in detail, it is conceivable that the therapeutic use of α_4-integrin blocking antibodies may inhibit pDC migration into various inflamed tissues.

Finally, the interstitial migration of pDCs deep within organs has not been elucidated and the cross-talk of pDCs with other cells, in particular NK cells, B cells, T cells and mDCs awaits further clarity. We plan to visualize pDC interactions with those cells by two-photon microscopy, which will illuminate the choreographic properties of pDCs in orchestrating immune responses. We anticipate that these further studies will broaden our knowledge about the role of pDCs in the immune system. The analysis of the precise functions of pDCs in various other clinical diseases will mark an important step towards developing novel therapeutics.