Plasmacytoid Dendritic Cells Are Dispensable during Primary Influenza Virus Infection

Amaya I. Wolf, Darya Buehler, Scott E. Hensley, Lois L. Cavanagh, E. John Wherry, Philippe Kastner, Susan Chan and Wolfgang Weninger

*J Immunol* 2009; 182:871-879; doi: 10.4049/jimmunol.182.2.871
http://www.jimmunol.org/content/182/2/871

**References**  This article cites 32 articles, 21 of which you can access for free at: http://www.jimmunol.org/content/182/2/871.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Plasmacytoid Dendritic Cells Are Dispensable during Primary Influenza Virus Infection

Amaya I. Wolf,* Darya Buehler,* Scott E. Hensley,† Lois L. Cavanagh,‡§ E. John Wherry,* Philippe Kastner,§ Susan Chan,§ and Wolfgang Weninger2*‡

Plasmacytoid dendritic cells (pDC) are thought to be pivotal in the first line of defense against viral infections. Although previous studies have suggested that pDC regulate the immune response against respiratory syncytial virus, their role in pulmonary infection with influenza virus has remained unclear. Using mice with GFP-tagged pDC, we observed a marked increase in pDC numbers in the lung airways 3 days after intranasal infection with influenza virus A/PR/8/34. To further investigate their potential involvement in the disease, we made use of pDC-deficient IkarosL/L mice. In the absence of pDC, the recruitment of T cells to the bronchoalveolar space was delayed, which could be reversed by the adoptive transfer of pDC before infection. Surprisingly, however, when compared with wild-type animals, IkarosL/L mice revealed a similar course of disease, as determined by weight loss, viral titers, levels of neutralizing Ab, and lung pathology. Moreover, the activation and differentiation of influenza-specific CD8+ effector T cells was unaltered in the absence of pDC, as was the generation of CD8+ memory T cells. Taken together, our study suggests that pDC regulate the accumulation of T cells in the bronchoalveolar space during early influenza virus infection, but are dispensable for the control of this disease. The Journal of Immunology, 2009, 182: 871–879.

Influenza is an acute febrile respiratory illness, caused by the infection of the respiratory tract with influenza virus A or B. Whereas the disease normally resolves within 1–2 wk in healthy individuals, potentially life-threatening complications cause substantial morbidity and mortality, especially in the elderly and in young children. It is thought that both the innate and adaptive immune systems contribute to the control of influenza virus infection. Infection results in the activation of dendritic cells (DC) leading to the subsequent stimulation of CD4+ and CD8+ T cells and the production of neutralizing Abs, which, together, are responsible for virus elimination. The precise nature of DC involved in the regulation of adaptive immune cells during influenza virus infection has, however, remained unclear. Given that these cells determine the quality of the adaptive immune responses and, therefore, may represent targets for the development of improved anti-influenza vaccines, it is imperative to define their relative contributions to the immune response against this virus.

DC can be divided into CD11c+ myeloid (conventional) DC (mDC) and CD11c− plasmacytoid DC (pDC) subsets. In the mouse, pDC are characterized by the expression of distinct phenotypic markers, i.e., CD11c+CD45R/B220+Ly6C/G+PDCA-1+120G8+ (1). pDC also express TLR 7 and 9, the pattern recognition receptors for viral single-stranded RNA and unmethylated DNA, respectively (2, 3). A characteristic feature of pDC is the rapid production of large amounts of type I IFNs following contact with certain viruses, including influenza virus. A major biologic effect of type I IFN is direct inhibition of viral replication. In addition, it has become increasingly clear that these cytokines also modulate the development of adaptive immunity by inducing mDC maturation and their capability of cross-presenting viral Ags, promoting plasma cell differentiation and enhancement of Ab production as well as isotype switching, and enhancing the survival of Ag-experienced T cells (reviewed in Ref. 4). In addition, pDC may directly cross-present viral Ags to T cells. They also produce a large variety of chemoattractants that may be involved in the recruitment of inflammatory cells to sites of pathogen invasion. Together, pDC act as early regulators of antiviral immunity.

Several studies have documented a role for pDC in mouse models of viral infection. For example, pDC are the prime source of type I IFNs during early (day 1.5) murine cytomegalovirus (MCMV) infection, as demonstrated by depletion of pDC with anti-Ly6C/G Ab (5). Recently, two studies demonstrated that depletion of pDC in respiratory syncytial virus (RSV) infection promoted pulmonary pathology and decreased viral clearance (6, 7). In contrast, Ab depletion studies of pDC had no affect on mouse morbidity and viral clearance after infection with the low pathogenic influenza virus strain X31 (8). Thus, the role of pDC in response to respiratory viral infections may differ between pathogens.

We have recently found that, as compared with CpG type B oligonucleotides, influenza A virus induces a unique transcriptional profile in pDC (9). We further showed that pDC activated by influenza virus in vitro showed characteristic high levels of type I
IFN production, but low expression of costimulatory molecules and decreased capacity to present Ag to naive T cells. The present study was undertaken to further investigate the role of pDC in influenza virus infection using a virus strain, influenza A/PR/8/34, that is highly pathogenic in mice. Following infection, we found that pDC accumulated in the respiratory tract early during disease. Making use of Ikaros<sup>L/L</sup> mice, which lack peripheral pDC, but harbor normal numbers of mDC (10, 11), we observed decreased recruitment of leukocytes to the airways. Nevertheless, Ikaros<sup>L/L</sup> mice mounted normal anti-influenza T and B cell responses, and recovered from disease similarly to wild-type animals. 

Materials and Methods

Mice

DPE-GFP mice that express GFP in all T cells and pDC, and Ikaros<sup>L/L</sup> mice lacking pDC have been described previously (10–13). Transgenic P14 mice carrying an MHC class I restricted TCR specific for GP<sub>33–41</sub> peptide from lymphocytic choriomeningitis virus (LCMV) GP were crossed to Thy1.1 congenic mice. All mice were housed and bred at the Wistar Institute under specific pathogen-free/viral Ab-free conditions. All studies were performed in accordance with Institutional Animal Care and User Committee guidelines at the Wistar Institute.

Influenza viruses and infection

Mouse-adapted influenza virus strain A/Puerto Rico/8/34 (H1N1) (PR8; a gift from Dr. Palese, Mount Sinai Hospital, New York, NY) and A/WSN/33 (WSN) were grown in the allantoic fluid of embryonated eggs. PR8-GP33 virus was generated by fusing the LCMV glycoprotein epitope 33–41 to the cytosolic part of neuraminidase of PR8 virus (a gift from Dr. Wehby, St. Jude Hospital, Memphis, TN). For infection, 4- to 6-wk-old mice were anesthetized i.p. with ketamine/xylazine (70 mg/7 mg per kg) and subsequently inoculated intranasally with 500 TCID<sub>50</sub> live virus (in 0.8 ml PBS/1% FBS via intranasal intubation). Spleens and lymph nodes (LN) were recovered from disease similarly to wild-type animals. 

Isolation of cells from the lungs, airways, and lymphoid tissues

Bronchoalveolar lavage (BAL) was performed on euthanized mice by flushing the airway compartment with 3 × 0.8 ml PBS/1% FBS via inserting a 18-gauge needle attached to a syringe into the trachea of mice. Lungs were perfused with 5 ml PBS/1% FBS through the right ventricle of the heart. Lungs were excised, cut into small pieces, and digested in HBSS supplemented with 400 U/ml Collagenase D (Roche) for 30 min at 37°C. Single cell suspensions were prepared by passing tissue pieces of the lungs, spleen and lymph nodes (LNs) through a metal wire mesh. RBC were lysed and samples were subjected to analysis by flow cytometry.

Expansion of pDC by Flt-3L

Three × 10<sup>6</sup> B16F10 melanoma cells modified to express Flt3-ligand (14) were injected into the neck skinfold of Ikaros<sup>L/L</sup> mice. Splenocytes were isolated from the spleens at 12–14 days after injection and were analyzed for the presence of pDC.

Determination of viral titers in lungs and virus-neutralizing Abs in sera of mice

The concentration of infectious virus in lungs was determined by titration of homogenized tissues in MDCK microcultures as previously described (15). To determine the levels of neutralizing Abs, sera of mice were heat inactivated and analyzed in a hemagglutination inhibition assay as previously described (16).

Lang histopathology

Lungs of naive or infected WT and Ikaros<sup>L/L</sup> mice were perfused with 250 mM HCl, and signals were measured based on their OD at 450 nm using a Microplate ELISA reader (Bio-Tek Instruments).

Statistical analysis

Data are presented as mean ± SEM. Statistical significance between two groups was calculated using the unpaired Student’s t test. Statistical significance between three groups was determined using one-way ANOVA with Bonferroni’s correction. All statistical tests were performed using the Prism software (GraphPad Software).

Results

PDC accumulate in the lungs and bronchoalveolar space in response to influenza virus infection

We have recently developed a transgenic mouse strain, DPE-GFP, in which T cells and pDC, but not B cells and mDC, express GFP (9, 12, 13). This is of advantage in situations where surface markers considered being “pDC-specific” under homeostatic conditions, for example identified by Abs 120G8 or mPDCA-1, are up-regulated on other leukocyte populations (17). This occurs primarily at sites of inflammation, and can confound the unequivocal identification of pDC using flow cytometry. To assess whether pDC are recruited to the lungs during influenza A virus infection, we inoculated anesthetized DPE-GFP mice intranasally with A/PR/8/34 virus. Under these conditions, mice develop an infection of the entire respiratory tract. As a measure of morbidity, we evaluated weight loss during infection over time. Similarly to wild-type C57BL/6 animals, weight loss peaked around day 10 p.i. in DPE-GFP mice, at which time point they had lost ~25% of their starting weight (Fig. 1A). Body weight returned to starting levels within 15 days p.i. Therefore, GFP expression did not alter the natural course of influenza in DPE-GFP mice.

We next identified pDC based on GFP expression and containing for CD45R/B220 or mPDCA-1. Although they were
Impaired expansion and differentiation of mPDCA-1+ pDC precursors from IkarosL/L mice in response to Flt-3L in vivo. A, PDC are greatly reduced in IkarosL/L mice. Single cell suspensions of wild-type (WT), Ikaros+/L, and IkarosL/L mice were analyzed for the presence of B220+ pDC. B–E, at different time points following infection with influenza virus A/PR/8/34. 

**FIGURE 1.** PDC numbers increase in lung and airways during influenza PR8 virus infection. DPE-GFP mice were infected intranasally with influenza virus A/PR/8/34. A, Weight curves of uninfected (■) and PR8 infected DPE-GFP mice (□) (n = 2–5 mice per time point). B–E, At different time points following infection with PR8 virus, single cell suspensions from bronchoalveolar space (BAL) (B), lungs (C), spleen (D) and mediastinal lymph nodes (E and F) were analyzed by flow cytometry for the presence of GFPhigh B220+ pDC and CD11chigh mDC (F). G, Percentage of CD40 and CD86 expression of GFPhigh B220+ pDC in lungs and BAL over time following PR8 infection.

Generally absent in the BAL fluid of uninfected mice, we observed a robust influx of pDC into the airways between 2 and 3 days, with a peak around day 7 p.i., and a sharp decline thereafter (Fig. 1B). Accumulation of pDC within the lung parenchyma revealed a more delayed course, with a 5-fold increase in numbers between days 7–15, followed by a decline at 3 wk p.i. (Fig. 1C). In contrast to the site of acute infection, pDC numbers in the spleen remained constant over the observation period (Fig. 1D). In lung-draining mediastinal lymph nodes (medLN), accumulation of pDC peaked around day 9 p.i. CD11chigh mDC showed more rapid recruitment to medLN as early as 3 days p.i. (6–8-fold increase over control) (Fig. 1, E and F).

PDC in the lungs and airways of influenza-infected DPE-GFP mice expressed elevated level of CD86, but not CD40 or CD80, between days 3 and 15 p.i. compared with pDC in lungs of noninfected controls (Fig. 1G and Ref. 9). In contrast, pDC retained a nonactivated phenotype in spleen and nondraining LNs during infection (data not shown).

Collectively, our data indicate that pDC are efficiently recruited into the lung and airways early during the anti-influenza response.
Together with previous findings showing the production of high amounts of type I IFNs as well as chemokines by pDC in response to influenza virus exposure in vitro, these results suggested that pDC are involved in the early regulation of anti-influenza immunity.

**Precursor pDC in Ikaros<sup>L/L</sup> mice do not differentiate into mature pDC after Flt-3L treatment in vivo**

To further investigate the role of pDC during influenza virus infection, it was necessary to create a situation where animals were devoid of pDC. Although Abs have been used for pDC depletion, results of this approach are variable and often incomplete. In addition, other cells expressing the same Ags may be affected. To circumvent this problem, we made use of Ikaros<sup>L/L</sup> mice (10, 11). Due to a hypomorphic mutation in the Ikaros gene, pDC in these mice are blocked in their differentiation in the bone marrow (BM), resulting in absence of pDC in secondary lymphoid and peripheral organs (Fig. 2A) (11). In comparison to BM of wild-type mice, pDC precursors from Ikaros<sup>L/L</sup> mice express some markers characteristic for pDC, such as the 120G8 Ag (11). However, they express little or no CD11c, are mainly Ly49Q<sup>−</sup>, and lack B220 expression. Most importantly, IFN-α production by BM pDC in Ikaros<sup>L/L</sup> mice in response to viruses was impaired, indicating that these cells are not functional (11).

MCMV infection of Ikaros<sup>L/L</sup> mice did not lead to the mobilization of mature pDC to the periphery, at least during the early stage of disease (1.5 days p.i.) (11). However, in this study, we were interested in following the course of influenza infection in Ikaros<sup>L/L</sup> mice for up to 3 wk. pDC precursors from Ikaros<sup>L/L</sup> BM express normal levels of the receptor for Flt-3L. Culture of unfractionated BM cells in the presence of Flt-3L led to the expansion of 120G8<sup>−</sup>CD11c<sup>−</sup> cells, however these cells failed to express B220 indicating that they do not terminally differentiate (11). To test whether the mPDCA<sup>−</sup> pDC-precursor present in the BM of Ikaros<sup>L/L</sup> mice exited into the periphery during Flt3L treatment in vivo, we implanted B16F10-tumor cells expressing recombinant Flt-3L into Ikaros<sup>L/L</sup> and wild-type mice, and enumerated pDC in various peripheral organs 2 wk later. Both mouse strains developed equally sized tumors (data not shown). Although the total cell numbers of spleens and lymph nodes were comparable between
the strains, mPDCA-1+ cells in IkarosL/L mice had expanded much less as compared with WT mice (Fig. 2B). Moreover, mPDCA-1+ cells remained mostly B220/CD11clow (Fig. 2C), indicating that mature pDC do not form in IkarosL/L mice even in the continuous presence of a growth factor. Therefore, because continuous Flt-3L treatment probably represents the strongest possible stimulus for pDC production, these mice should allow for investigating pDC-deficient conditions for prolonged periods of time.

We next assessed the functional consequences of the absence of mature pDC in IkarosL/L mice. Splenocytes from WT and IkarosL/L mice were cultured overnight in the presence or absence of UV-inactivated PR8 virus. In contrast to wild-type splenocytes, cells from IkarosL/L mice were deficient in IFN-α production (Fig. 3A). Although the percentage of CD11c+ mDC was comparable in the spleen of WT and IkarosL/L mice, IkarosL/L mice harbored slightly lower percentages of B cells after overnight culture in medium or with PR8 virus (WT: 48% vs IkarosL/L: 38%; Fig. 3B). Upon stimulation with PR8 virus, mDC and B cells up-regulated the costimulatory molecules CD40 and CD86 to a similar extent (Fig. 3B). In addition, other TLR ligands such as polyI:C and LPS induced normal activation of mDC from IkarosL/L mice (data not shown), indicating that the absence of pDC did not influence the activation of mDC and B cells after in vitro stimulation.

We then asked whether mature pDC could be detected in influenza virus-infected IkarosL/L mice. To this end, wild-type and IkarosL/L mice received influenza virus PR8 intranasally, and organs were analyzed at various time points thereafter (Fig. 3C, and data not shown). At all time points studied, no B220+CD11c+PDCA-1+ cells were found in spleens, lungs, or BAL fluid of IkarosL/L mice. We concluded that the pDC precursors were unable to differentiate into mature pDC in the periphery during infection, and that IkarosL/L mice represent a useful tool to further study the effects of their deficiency during this infection.

IkarosL/L mice respond to influenza virus infection similarly as wild-type animals

To determine whether the absence of pDC during influenza infection affected the course of disease, wild-type and IkarosL/L mice were infected intranasally with a sublethal dose of PR8 virus. As shown in Fig. 4A, weight loss of IkarosL/L mice was indistinguishable from wild-type animals, indicating that morbidity was similar between the strains. These data suggest that the overall course of influenza virus infection, including recovery, was normal in the absence of pDC. Next, we asked whether IkarosL/L mice exhibited delayed viral clearance. Although 4 days p.i., IkarosL/L mice had slightly reduced viral titers as compared with wild-type animals, there was no statistically significant difference at day 8 p.i. (Fig. 4B). By day 10 p.i., infectious virus in lungs of either wild-type or IkarosL/L mice was undetectable (data not shown). It has previously been shown that virus-stimulated pDC contribute to B cell differentiation into plasma cells, and that depletion of pDC from human PBL.
abrogated production of anti-influenza Abs (18). When we analyzed the serum from animals at day 39 p.i., we found that Ikaros<sup>L/L</sup> mice had similar levels of neutralizing Ab titers as compared with wild-type mice (Fig. 4C). In addition, histopathology of lungs at day 10 p.i. showed a qualitatively similar inflammatory infiltrate (Fig. 4D).

To test the possibility that the immune defense against a influenza A virus strain with higher disseminating activity relies on pDC, we infected mice with influenza WSN/33. This strain is unusual in that the plasminogen-binding activity of neuraminidase can lead to proteolytic cleavage of hemagglutinin in multiple organs (19). Moreover, it was recently demonstrated that pDC produce IFN-α and -β in response to WSN-infection in vivo (20), suggesting that they may contribute to clearance of this virus. However, as with PR8 virus, Ikaros<sup>L/L</sup> mice revealed similar weight loss curves and recovery from infection as wild-type animals, and also comparable WSN titers in lungs (Fig. 4, E and F).

Together, our results indicate that in the absence of pDC, mice clear influenza A viruses as efficiently as wild-type mice, and also mount similar B cell responses.

**Ikaros<sup>L/L</sup> mice have impaired early T cell recruitment to the airways during influenza virus infection**

PDC are among the earliest inflammatory cells to enter the bronchoalveolar space following influenza A virus infection. In addition, we have previously shown that these cells produce large amounts of chemotactants, including XCL1 and CXCL10 (9). Thus, we hypothesized that they may be involved in effector cell recruitment to this anatomical compartment (21, 22). As depicted in Fig. 5A, 4 days p.i. with PR8 virus, BAL fluid of Ikaros<sup>L/L</sup> mice contained 3-fold lower total leukocyte numbers compared with wild-type mice (Fig. 5A). In particular, CD3<sup>+</sup> T cells were reduced (Fig. 5B), while neutrophil numbers were unaffected (Fig. 5C). At day 8 p.i., no difference in total leukocyte nor CD3<sup>+</sup> T cell numbers was observed (Fig. 5, D and E). To test whether reconstitution of Ikaros<sup>L/L</sup> mice with pDC could increase the number of cells in the bronchoalveolar space, mice received 2–3 × 10<sup>8</sup> freshly isolated pDC one day before influenza virus infection. This treatment reversed the defect in cell recruitment (Fig. 5, A and B). These data indicate that pDC are important for the efficient early recruitment of T cells into BAL during influenza virus A infection, possibly through the production of chemokines (21, 22). Nevertheless, compensatory mechanisms likely exist, as no difference in recruitment was observed at later stages of disease.

**Influenza-specific effector and memory T cell differentiation is unaltered in Ikaros<sup>L/L</sup> mice**

Previous reports in a herpes virus infection model have shown that depletion of pDC results in impaired effector CTL generation (23). We therefore investigated the quality of the effector and memory CD8<sup>+</sup> T cell response in Ikaros<sup>L/L</sup> mice during influenza virus infection. We made use of a modified PR8 virus expressing the LCMV glycoprotein epitope GP<sub>33-41</sub> (PR8-GP33), which is recognized by CD8<sup>+</sup> T cells from transgenic P14 mice. We adoptively transferred CFSE-labeled P14<sup>+</sup>Thy1.1 cells into wild-type and Ikaros<sup>L/L</sup> mice (Thy1.2<sup>+</sup>) to follow their proliferation. Whereas CD8<sup>+</sup> T cells in uninfected mice remained undivided (Fig. 6A), at day 4 p.i. CD8<sup>+</sup> P14 T cells had undergone multiple divisions in both wild-type and Ikaros<sup>L/L</sup> mice infected with PR8-GP33. Eighty to ninety percent of CD8<sup>+</sup> P14 T cells showed an activated phenotype evidenced by up-regulation of CD25 and CD69 (data not shown). Similar results were obtained using the WSN strain expressing OVA and CD8<sup>+</sup> T cells specific for OVA from OT1 mice (data not shown). We also tested whether P14 CD8<sup>+</sup> T cells acquired similar effector functions in wild-type or Ikaros<sup>L/L</sup> mice. As shown in Fig. 6B, an equal percentage of CD8<sup>+</sup> P14 T cells in the spleen produced IFN-γ. Similar results were obtained in cells isolated from the lungs (data not shown).

Because the precursor frequency of naive Ag-specific T cells is artificially increased in the adoptive transfer model, we also assessed the generation of the endogenous Ag-specific effector and memory CD8<sup>+</sup> T cells. Using MHC class I tetramers for the immunodominant NP<sub>366–374</sub> peptide of PR8, we found a similar percentage of virus-specific effector T cells in the spleen and lungs of Ikaros<sup>L/L</sup> and wild-type mice 10 days p.i. (Fig. 6C). Similarly, the percentage and total numbers of NP<sub>366–374</sub> memory CD8<sup>+</sup> T...
cells were comparable in Ikaros<sup>L/L</sup> and wild-type mice 39 days p.i. (Fig. 6, D and E).

Our results suggest that pDC are not critical for the generation of Ag-specific effector and memory CD8<sup>+</sup> T cells during PR8 virus infection.

**Discussion**

In vitro studies have shown that pDC readily respond to exposure of influenza virus by the secretion of proinflammatory mediators, IFNs, and chemokines. Further, during respiratory infection with certain viruses, pDC have been found to produce type I IFNs within the lungs of infected mice (24). This study was designed to test the role of pDC in influenza virus infection using pDC-deficient mice. We found that pDC were not essential for viral clearance, the formation of neutralizing Abs or the generation of Ag-specific effector and memory CD8<sup>+</sup> T cells. Nevertheless, pDC participated in lymphocyte recruitment to the airways during early stages of disease.

To analyze the precise role of pDC during influenza infection, ideally mice have to be rendered pDC deficient. This can be achieved by two principal approaches, i.e., Ab depletion or genetic modification of mice. Abs have been used widely, but this carries the risk of incomplete depletion and/or unwanted effects on non-pDC populations. In this study, we made use of a mutant mouse strain, Ikaros<sup>L/L</sup>, in which pDC are absent in spleen, lungs, and lymph nodes (11). Ikaros<sup>L/L</sup> mice express low levels of functional Ikaros protein in hematopoietic cells (10), and this mutation arrests pDC development in the BM (11). PDC-deficient Ikaros<sup>L/L</sup> mice have slightly reduced B cell and blood neutrophil numbers. In addition, these mice lack certain LNs, including the inguinal and medLN, while others, such as the cervical LN, are present. Nevertheless, even mice that lack all secondary lymphoid organs generate anti-influenza effector and memory T cell responses comparably to wild-type mice, and produce neutralizing Abs (25). Therefore, while keeping the shortcomings of Ikaros<sup>L/L</sup> mice in mind, they represent a useful alternative approach to study the effects of pDC absence in immune responses.

We found no difference in morbidity of Ikaros<sup>L/L</sup> mice in response to intranasal infection with a sublethal dose of live influenza virus as compared with WT mice (Fig. 4A). Moreover, Ikaros<sup>L/L</sup> mice recovered in a similar fashion with no significant prolonged increase in viral titers in the lungs (Fig. 4B). These data
are consistent with the recent observation that mice treated with 120G8 mAb for pDC depletion had the same viral burden in the lungs after X31 infection as untreated mice (8). Interestingly, IFN-α levels in lungs and BAL of the mice were unaffected in the latter study suggesting that production of this cytokine may be independent of pDC. Moreover, previous studies using mice that are unable to respond to type I IFNs, i.e., IFN-α-receptor knockout mice (26), showed that viral titers in lungs were not significantly different from wild-type controls following infection with influenza PR8 virus. We demonstrated additionally that recovery of Ikaros<sup>L/L</sup> mice infected with WSN virus was similar to WT mice (Fig. 4E). We hypothesize that other cells, such as alveolar macrophages and/or virally infected epithelial cells produce type I IFN (24, 27), and may therefore compensate for the lack of pDC-derived IFNs during influenza infection. We wish to point out that a potential caveat with the interpretation of our results is that we used sublethal infection doses for all of our experiments. Thus, while under these conditions, pDC were found to be dispensable for virus clearance, it is still conceivable that pDC-derived chemokines play a more prominent function during lethal infection with very high doses of virus or with more virulent strains.

We observed that the lack of peripheral pDC in Ikaros<sup>L/L</sup> mice resulted in reduced recruitment of leukocytes into the bronchoalveolar space early upon infection, while neutrophil numbers were unaffected (Fig. 5). Cell recruitment could be restored to normal levels when Ikaros<sup>L/L</sup> mice were reconstituted with pDC before the infection, suggesting that the initial influx of T cells into BAL is regulated by pDC. Due to the fact that pDC produce a variety of chemokines, such as CXCL9, CXCL10, CXCL11, and CCL4 (21, 22) upon stimulation, it seems likely that pDC support the recruitment of CCR5<sup>+</sup> and CXCR3<sup>+</sup> effector T cells to the lung during infection. It has been shown that the levels of various chemokines, including CCL5 (RANTES) and CXCL10 (IP-10), ligands for CCR5 and CXCR3, respectively, are increased in the lungs between days 3–15 following viral infection (28). The comparable number of BAL leukocytes at day 8 p.i. suggests that pDC deficiency can be overcome at later stages of infection. It has been shown that upon depletion of pDC, other cells could be induced to produce higher levels of cytokines such as IL-12 to compensate for a lack of function of pDC during MCMV infection (5). Similar mechanisms could be envisioned for chemokines.

To test the effects of pDC deficiency on effector T cell differentiation, we transferred CFSE-labeled T cells specific for the LCMV peptide GP<sub>33–41</sub> into wild-type and Ikaros<sup>L/L</sup> mice. Following infection with recombinant virus PR8-GP31, we observed that Ag-specific donor cells proliferated equally well in both recipient mouse strains (Fig. 6A). These results are consistent with previous studies, which have suggested that pDC are not the major APCs following influenza virus infection (29, 30) despite the fact that type I IFNs are thought to act and promote CD8<sup>+</sup> T cell cross-priming and differentiation (31). Neither in IFNAR-knockout mice (26), in pDC-depleted mice (8) nor in Ikaros<sup>L/L</sup> mice were effector CD8<sup>+</sup> T cells diminished or showed altered cytokine production (Fig. 6B). In addition, pDC isolated from medLN of infected animals were unable to induce significant proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (8, 32). Together, these results suggest that pDC are not involved in CD8<sup>+</sup> T cell priming during influenza virus infection.

Furthermore, we found that pDC matured incompletely in lung-draining medLN, as they only partially up-regulated costimulatory molecules. In contrast, mature CD11c<sup>Bright</sup> mDC rapidly increased in numbers between 24 and 72 h post infection. Also, the lack of pDC did not, at the phenotypic level, significantly influence the maturation of mDC in Ikaros<sup>L/L</sup> mice. These data are consistent with previous results showing that IL-12 levels were unaltered in Ikaros<sup>L/L</sup> mice following injection of various TLR ligands (11). However, previous reports in an HSV infection model have shown that pDC provide help for the Ag-presenting capacity of mDC (23). Therefore, pDC differ in their accessory function for mDC depending on the infectious agent.

Another observation was the fact that influenza-infected Ikaros<sup>L/L</sup> mice mounted normal neutralizing Ab responses (Fig. 4C). These results are in contrast to a recent study showing that hemagglutination inhibition-titers in mice treated with the 120G8 Ab are reduced (8). Although other studies of human pDC indicated that these cells induce B cell responses in an IFN-α-dependent manner, the study by GeurtsvanKessel (8) reported that IFN-α levels are normal despite pDC-depletion, which may suggest that the depleting mAb could directly or indirectly act on B cells. This is supported by the original observation that the Ag recognized by the 120G8 Ab is up-regulated on activated B cells and DC (17).

Previous studies have demonstrated a role of pDC in murine respiratory syncytial virus infection. Thus, pDC depletion during RSV-infection resulted in increased pulmonary pathology, and adoptive transfer of pDC promoted viral clearance (6, 7). Another study suggests that type I IFN production of pDC in RSV-infection may not be a critical factor and indicates that pDC may have limited contributions in RSV-infected mice (20). To demonstrate that pDC can enhance viral elimination, Wang et al. (6) activated bone marrow-derived pDC with CpG 1826 before intratracheal transfer into animals that were subsequently infected with RSV (7). However, transcriptome analysis comparing pDC activated with CpG 1826 or PR8 virus have shown that these stimuli induce vastly different programs in these cells (9). CpG 1826-activated pDC expressed higher levels of costimulatory molecules and produced much higher amounts of proinflammatory cytokines such as TNF-α, IL-12, and IL-6. An exception was type I IFNs, which were higher in PR8-stimulated pDC. Therefore, comparisons between differentially activated pDC populations have to be interpreted with care. Consequently, further insight into the distinct actions of pDC against viral lung infections is warranted.

In summary, we have demonstrated that pDC functions during a primary immune response to influenza virus infection seem to be limited to the support of T cell recruitment into the bronchoalveolar space. PDC appear to be dispensable for the clearance of virus, priming and differentiation into effector/memory CD8<sup>+</sup> T cells as well as the production of virus-neutralizing Abs. Thus, the results of this study imply that pDC may be dispensable for the recovery from a primary infection with influenza PR8.

Acknowledgments

We thank Krystyna Mordzanowska for excellent technical support with viral assays, Russel Delgiacco for help with lung histology, and Drs. Peter Palese and Richard Webby for providing virus strains. We also thank Dr. Jan Erikson and members of the Weninger laboratory for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References
