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# Mouse CD8 $\alpha^+$ DCs and human BDCA3 $^+$ DCs are major producers of IFN- $\lambda$ in response to poly IC

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**Polyinosinic:polycytidylic acid (poly IC), a double-stranded RNA, is an effective adjuvant in vivo. IFN- $\lambda$ s (also termed IL-28/29) are potent immunomodulatory and antiviral cytokines. We demonstrate that poly IC injection in vivo induces large amounts of IFN- $\lambda$ , which depended on hematopoietic cells and the presence of TLR3 (Toll-like receptor 3), IRF3 (IFN regulatory factor 3), IRF7, IFN-I receptor, Fms-related tyrosine kinase 3 ligand (FL), and IRF8 but not on MyD88 (myeloid differentiation factor 88), Rig-like helicases, or lymphocytes. Upon poly IC injection in vivo, the IFN- $\lambda$  production by splenocytes segregated with cells phenotypically resembling CD8 $\alpha^+$  conventional dendritic cells (DCs [cDCs]). In vitro experiments revealed that CD8 $\alpha^+$  cDCs were the major producers of IFN- $\lambda$  in response to poly IC, whereas both CD8 $\alpha^+$  cDCs and plasmacytoid DCs produced large amounts of IFN- $\lambda$  in response to HSV-1 or parapoxvirus. The nature of the stimulus and the cytokine milieu determined whether CD8 $\alpha^+$  cDCs produced IFN- $\lambda$  or IL-12p70. Human DCs expressing BDCA3 (CD141), which is considered to be the human counterpart of murine CD8 $\alpha^+$  DCs, also produced large amounts of IFN- $\lambda$  upon poly IC stimulation. Thus, IFN- $\lambda$  production in response to poly IC is a novel function of mouse CD8 $\alpha^+$  cDCs and their human equivalents.**

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Abbreviations used: cDC, conventional DC; DN, double negative; dsRNA, double-stranded RNA; DT, diphtheria toxin; DTR, DT receptor; FL, Fms-related tyrosine kinase 3 ligand; IFN-IR, IFN-I receptor; IRF, IFN regulatory factor; ODN, oligonucleotide; pDC, plasmacytoid DC; poly IC, polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; RLH, Rig-like helicase; TLR, Toll-like receptor.

The IFN- $\lambda$ 1, IFN- $\lambda$ 2, and IFN- $\lambda$ 3 cytokine family, also called IL-29, IL-28A, and IL-28B, respectively, has recently been identified (Kotenko et al., 2003; Sheppard et al., 2003) and shown to be related to type I IFNs (IFN-Is) and the IL-10 family of cytokines. IFN- $\lambda$ s signal via a heterodimeric receptor, consisting of one chain unique for IFN- $\lambda$  (IFN- $\lambda$ R1 or IL-28R $\alpha$ ) and another chain (IL-10R2), which is shared with IL-10-related cytokines. Similar to IFN-Is, IFN- $\lambda$ s possess antiviral, antitumor, and various immune-modulating functions (Li et al., 2009). In contrast to the ubiquitous expression of the IFN-I receptor (IFN-IR), the expression

of the IFN- $\lambda$  receptor is restricted to certain cell types, including epithelial cells and plasmacytoid DCs (pDCs; Ank et al., 2008; Sommereyns et al., 2008). Exposure to viruses or analogues of nucleic acids such as CpG-oligonucleotide (ODN), conditions known to trigger the production of IFN-Is, also induces IFN- $\lambda$ s and largely depends on similar signaling components (Coccia et al., 2004; Onoguchi et al., 2007; Osterlund

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et al., 2007; Ank et al., 2008). The precise role of IFN- $\lambda$ s in viral infections is not yet known. However, it plays a role in Toll-like receptor (TLR)-induced protection against mucosal viral infections, and recent studies link the *IL28B* gene with an ability to clear and recover from Hepatitis C infection (Ank et al., 2008; Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009). Thus, it is of utmost importance to understand the cellular origin of IFN- $\lambda$ s and the regulation of its production.

Several cell types, including PBMCs, monocyte-derived DCs, and pDCs, stimulated with various agents in vitro have been described to produce IFN- $\lambda$ , mostly analyzed by gene transcripts (Kotenko et al., 2003; Sheppard et al., 2003; Coccia et al., 2004; Osterlund et al., 2005; Ank et al., 2008). Polyinosinic: polycytidylic acid (poly IC) is used as an important immune stimulant and is an excellent adjuvant for the induction of T<sub>H</sub>1 CD4 T cell responses in a DC-targeted vaccine model (Schulz et al., 2005; Longhi et al., 2009). It is a mimic of viral double-stranded RNA (dsRNA) and is recognized by TRIF-dependent TLR3 or Cardif (also known as IPS-1, MAVS, and VISA)-dependent Rig-like helicases (RLHs). Injection of poly IC in vivo induces large systemic levels of IFN- $\alpha$  (Field et al., 1967). Nonhematopoietic cells using the RLH MDA5 have been identified to be responsible for the majority of this systemic IFN- $\alpha$  (Gitlin et al., 2006; Longhi et al., 2009).

IFN-I production in response to RLH or TLR signaling depends on the presence and the activation of transcription factors of the IFN regulatory factor (IRF) family. IRF3 and IRF7 have been described to be important for the optimal production of IFN-I (Tamura et al., 2008). However, depending on the cellular source and the pattern recognition receptor (PRR) used, the role of individual IRFs varies (Tamura et al., 2008). For the production of IFN- $\lambda$ s, much less is known, but a role for IRF3 and IRF7 has been suggested for different stimuli and different cells, including DCs (Coccia et al., 2004; Onoguchi et al., 2007; Osterlund et al., 2007).

Based on phenotypic, functional, and developmental differences, the DCs of the spleen are divided into several subsets. A major distinction can be made between pDCs and conventional DCs (cDCs). pDCs are best known for their outstanding capacity for IFN- $\alpha$  production. cDCs are further grouped into CD8 $\alpha$ <sup>+</sup> and CD8<sup>-</sup> cDC subsets (Vremec et al., 1992). The CD8<sup>-</sup> cDCs can be further segregated by the expression of CD4 into CD4<sup>+</sup> cDCs and double negative (DN) cDCs (Vremec et al., 2000), but other surface markers including CD11b and CD172a are similarly highly expressed by CD4<sup>+</sup> and DN cDCs. Quantitative proteomic analysis of spleen cDC subsets clearly demonstrated the relatedness of the two CD8<sup>-</sup> cDC subsets distinguishing these DCs from CD8 $\alpha$ <sup>+</sup> cDCs (Luber et al., 2010).

CD8 $\alpha$ <sup>+</sup> cDCs are well known for the production of IL-12p70 in various organs, including spleen, lymph nodes, thymus, and liver (Reis e Sousa et al., 1997; Hochrein et al., 2001; Pillarisetty et al., 2004). Another important function of CD8 $\alpha$ <sup>+</sup> cDCs is their outstanding capacity for cross-presentation (Shortman et al., 2009). Cross-presentation is not confined to

spleen cDCs bearing the CD8 $\alpha$  molecule because a rare subset of CD8<sup>-</sup> cDCs with the cross-presenting ability of CD8 $\alpha$ <sup>+</sup> cDCs has been identified in spleen. They lack CD11b and CD172a expression and express CD205 and high levels of CD24 (Bedoui et al., 2009). Upon treatment with Fms-related tyrosine kinase 3 ligand (FL), the numbers of those equivalent of CD8 $\alpha$ <sup>+</sup> (eCD8 $\alpha$ ) cDCs can be greatly enhanced in vivo (Bedoui et al., 2009). Likewise, FL treatment of BM cells can drive eCD8 $\alpha$  cDCs (as well as eCD8<sup>-</sup> cDCs and pDCs) in vitro. Recent work on mice deficient for, or carrying mutations in, transcription factors such as IRF8 or Batf3 demonstrated the absence of CD8 $\alpha$ <sup>+</sup> cDCs in lymphoid organs and lack of eCD8 $\alpha$  cDCs in peripheral organs as well as the failure to drive eCD8 $\alpha$  cDCs in FL cultures (Schivavoni et al., 2002; Aliberti et al., 2003; Hildner et al., 2008; Edelson et al., 2010). Recently, cross-presenting CD8<sup>-</sup> cDCs in other organs such as lymph nodes have also been characterized (Henri et al., 2010).

CD8 is not expressed on human DCs, whereas CD4 is expressed on all DC subsets. A set of antibodies designated BDCA1–4 has been developed to differentiate between pDCs and subsets of cDCs (Dzionek et al., 2000). The human BDCA3-positive cDCs have been proposed as the human eCD8 $\alpha$  cDCs. Common to the mouse CD8 $\alpha$ <sup>+</sup> DCs, BDCA3-positive cDCs selectively express high levels of Clec9a and Necl2 but low amounts of CD11b (Shortman et al., 2009). Genome-wide transcriptional analysis substantiated a close relationship of murine CD8 $\alpha$ <sup>+</sup> cDCs with human BDCA3<sup>+</sup> cDCs (Robbins et al., 2008). Like mouse CD8 $\alpha$ <sup>+</sup> and eCD8 $\alpha$  cDCs, human BDCA3<sup>+</sup> cDCs have been found in various organs, including blood, spleen, tonsils, lymph nodes, and liver (Dzionek et al., 2000; Lindstedt et al., 2005; Velásquez-Lopera et al., 2008; Bamboat et al., 2009; Poulin et al., 2010). Recently, the functional hallmarks of murine CD8 $\alpha$ <sup>+</sup> cDCs, IL-12p70 production, cross-presentation ability, and TLR3 expression, have also been shown to align with human BDCA3<sup>+</sup> cDCs. This strongly suggests that BDCA3<sup>+</sup> cDCs are the human equivalents of murine CD8 $\alpha$ <sup>+</sup> cDCs (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010; Villadangos and Shortman, 2010).

Expression of the different nucleic acid-sensing systems TLR3, TLR7, or TLR9 and the RLHs varies among DC subsets (Hochrein and O’Keeffe, 2008). The downstream functions after engagement of these receptors also differ among the different DCs. pDCs predominantly use TLR7 and TLR9 for nucleic acid sensing, resulting in the high production of IFN-I and IFN- $\lambda$ s. Among cDCs, CD8 $\alpha$ <sup>+</sup> cDCs highly express TLR3 but lack expression of TLR7 (Edwards et al., 2003) and, in stark contrast to CD8<sup>-</sup> cDCs, hardly express the RLHs and as a consequence are unable to detect the single-stranded RNA viruses Sendai or influenza virus (Luber et al., 2010).

DNA viruses such as poxviruses or herpesviruses are recognized by pDCs predominantly via a TLR9- and MyD88 (myeloid differentiation factor 88)-dependent pathway, whereas other cells including cDCs use MyD88-independent, poorly defined recognition pathways (Lund et al., 2003; Hochrein et al., 2004;

Krug et al., 2004; Samuelsson et al., 2008; Siegemund et al., 2009; Wilkins and Gale, 2010).

In this study, we found that poly IC induced systemic production of IFN- $\lambda$ , and we investigated the molecular and cellular events required for this production. Mice that lacked CD8 $\alpha^+$  cDCs were unable to produce IFN- $\lambda$  to poly IC in vivo. This production depended on TLR3, IRF3, IRF7, and IFN-IR. We identified mouse CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs as the major producers of IFN- $\lambda$  in response to poly IC in vitro. Similar to mice, among human cDCs, human eCD8 $\alpha$  cDCs isolated via expression of BDCA3 were found to produce large quantities of IFN- $\lambda$ s upon stimulation with poly IC. Thus, IFN- $\lambda$  production to poly IC was identified as a hallmark function of eCD8 $\alpha$  cDCs in mice and man.

## RESULTS

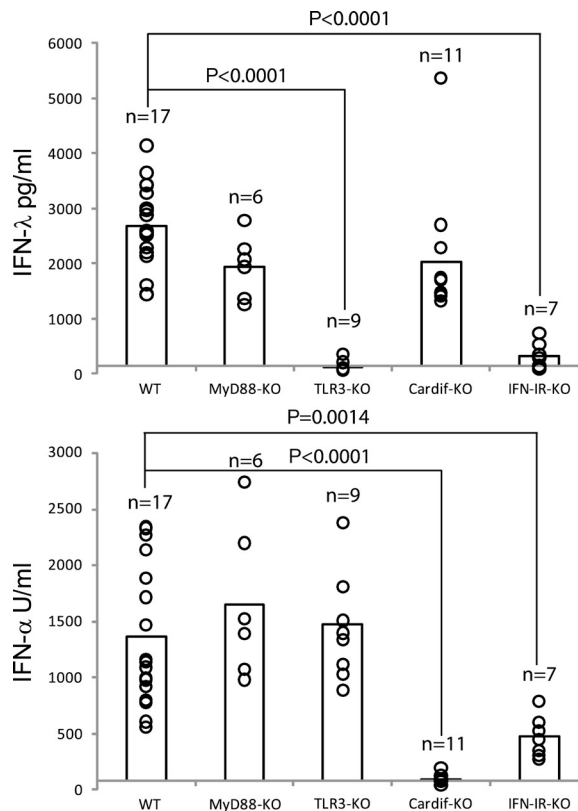
### Poly IC induces systemic IFN- $\lambda$ production in vivo, which depends on TLR3 and IFN-IR

Poly IC is sensed by redundant pathways, and roles for RLHs as well as TLR3 have been described previously (Alexopoulou et al., 2001; Gitlin et al., 2006). Injection of poly IC is known to induce large systemic levels of IFN- $\alpha$ . In this study, we found that the injection of poly IC in WT mice also induced large quantities of IFN- $\lambda$  (Fig. 1). To determine the PRRs involved in this IFN- $\lambda$  production, we injected poly IC into mice deficient for various PRRs or their adaptor molecules, specifically TLR3, MyD88, or Cardif (also known as MAVS, IPS-1, or VISA), and measured IFN- $\lambda$  as well as IFN- $\alpha$  in the corresponding sera (Fig. 1). Large amounts of IFN- $\lambda$  and IFN- $\alpha$  were induced in WT and MyD88-KO mice (Fig. 1), demonstrating that MyD88-dependent TLRs were not involved. This also suggests that pDCs, which largely depend on MyD88 for IFN production, unlikely contributed to the production of both cytokines under those conditions. However, deficiency of TLR3 resulted in abrogated IFN- $\lambda$  production with no effect on the production of IFN- $\alpha$  (Fig. 1). In contrast, Cardif deficiency revealed no effects on IFN- $\lambda$  production but, consistent with previous studies, using MDA5-deficient mice, complete abrogation of serum IFN- $\alpha$  (Fig. 1; Gitlin et al., 2006; Longhi et al., 2009). Thus, whereas poly IC induced large systemic levels of both IFN- $\lambda$  and IFN- $\alpha$  in WT mice, the dependence on TLR3 or Cardif for their production seems to be mutually exclusive.

It has been described that optimal IFN- $\alpha$  production to poly IC in vivo requires expression of a functional IFN-IR (Barchet et al., 2002). A role for IFN-IR has also been proposed for the production of IFN- $\lambda$  in response to viruses (Ank et al., 2008). In this study, we found that systemic production of IFN- $\lambda$  and IFN- $\alpha$  in response to poly IC was largely dependent on the presence of the IFN-IR (Fig. 1).

### IFN- $\lambda$ production to poly IC in vivo depends on IRF3 and IRF7

To get an insight into the transcription factors involved in the IFN- $\lambda$  production to poly IC in vivo, we challenged mice deficient for either IRF3 or IRF7 or both IRF3+7 (Fig. 2).

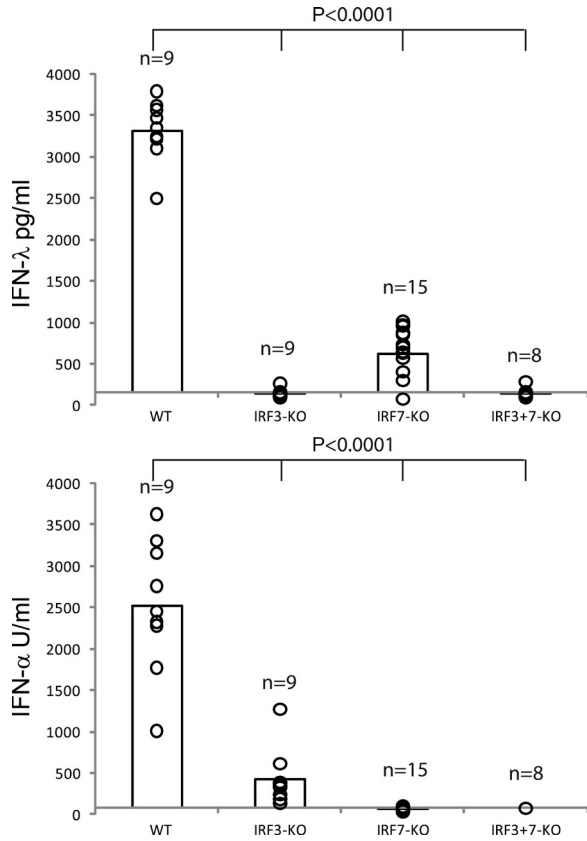


**Figure 1. Poly IC-induced IFN- $\lambda$  production in vivo depends on TLR3 and IFN-IR but not on MyD88 or Cardif.** Mice with the indicated genotype were injected i.v. with 100  $\mu$ g poly IC. After 3–4 h, sera were analyzed for IFN- $\lambda$  and IFN- $\alpha$ . Circles indicate the results of individual mice, and their total number (n) is indicated in the graph. The bars represent the mean of all mice per genotype. At least three independent experiments have been performed.

Mice that lacked IRF3 had a completely abrogated IFN- $\lambda$  production and an 80% reduction in the production of IFN- $\alpha$ . Mice lacking IRF7 showed the inverse pattern with a complete lack of IFN- $\alpha$  production and an 80% reduction in the IFN- $\lambda$  production. Mice deficient for both transcription factors (IRF3+7) produced neither IFN- $\lambda$  nor IFN- $\alpha$ . Thus, the full production of poly IC-induced IFN- $\lambda$  and IFN- $\alpha$  in vivo depended on the presence of IRF3 and IRF7 (Fig. 2).

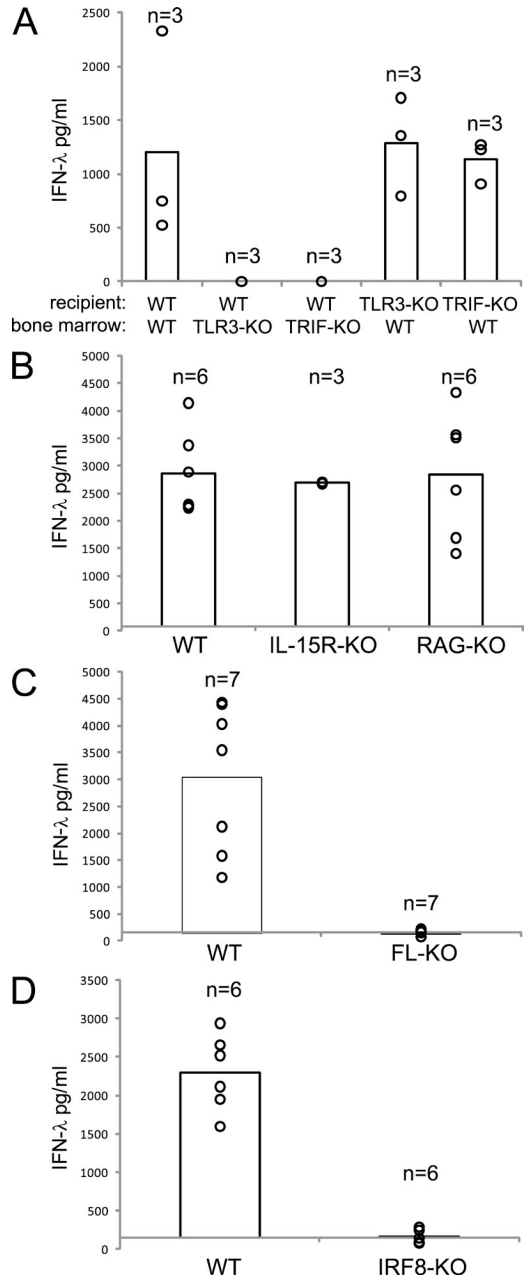
### IFN- $\lambda$ production to poly IC in vivo depends on hematopoietic cells, FL, and IRF8 but not on lymphocytes

Systemic IFN- $\alpha$  in response to poly IC originates from non-hematopoietic cells via MDA5 recognition (Gitlin et al., 2006; Longhi et al., 2009). To test for the origin of systemic IFN- $\lambda$ , we made BM chimeras of WT and TLR3 or TRIF mice. The data show that TLR3-KO and TRIF-KO mice, which received a WT BM, gained systemic IFN- $\lambda$ -producing capacity upon poly IC injection (Fig. 3 A). In contrast, WT mice reconstituted with TLR3-KO or TRIF-KO BM were unable to produce systemic IFN- $\lambda$  to poly IC (Fig. 3 A). Thus, hematopoietic cells are the origin of this IFN- $\lambda$ .



**Figure 2. IFN-λ production to poly IC in vivo depends on IRF3 and IRF7.** Mice with the indicated genotype were injected i.v. with 100 μg poly IC. After 3–4 h, sera were analyzed for IFN-λ and IFN-α. Circles indicate the result of individual mice, and their total number (n) is indicated in the graph. The bars represent the mean of all mice per genotype. Three independent experiments have been performed.

To rule out lymphocytes as a source of the systemic IFN-λ production in vivo, we tested IL-15R-KO and RAG-KO mice, which lack NK cells and B cells and T cells, respectively, but found that both strains were able to produce normal WT levels of IFN-λ (Fig. 3 B) and IFN-α (Fig. S4 A). To identify the cellular source of the systemic IFN-λ, we tested FL-KO mice, which are known to have greatly reduced numbers of DCs (McKenna et al., 2000). In contrast to WT mice, poly IC-injected FL-KO mice were unable to produce IFN-λ (Fig. 3 C), whereas IFN-α was produced (Fig. S4 B), suggesting a role for DCs in the production of IFN-λ to poly IC in vivo. Application of recombinant FL into FL-KO mice not only restored but even increased their IFN-λ-producing capacity above the WT level (unpublished data). Along those lines, FL-treated WT mice, which display elevated DC numbers, skewed toward increased CD8α<sup>+</sup> cDCs and eCD8α cDCs (O’Keeffe et al., 2002; Bedoui et al., 2009), had a greatly increased systemic IFN-λ response to poly IC challenge (unpublished data). The FL dependence suggests that IFN-λ production to poly IC in vivo is largely mediated by DCs.



**Figure 3. IFN-λ production to poly IC in vivo depends on hematopoietic cells, FL, and IRF8.** Mice with the indicated genotype were injected i.v. with 100 μg poly IC, and after 3–4 h, sera were analyzed for IFN-λ. (A) BM reconstituted mice as indicated. (B) WT, IL-15R-KO, and RAG1-KO. (C) WT and FL-KO. (D) WT and IRF8-KO. Circles indicate the result of individual mice, and their total number (n) is indicated in the graph. The bars represent the mean of all mice per genotype. One (BM chimeras in A and IL-15R-KO in B), two (WT and RAG-KO in B and WT and IRF8-KO in D), and three (WT and FL-KO in C) independent experiments have been performed.

To clarify which DC subset would be the source of IFN-λ production to poly IC in vivo, we used mice lacking the transcription factor IRF8 and as a consequence CD8α<sup>+</sup> and eCD8α cDCs (Schiavoni et al., 2002; Aliberti et al., 2003;



Edelson et al., 2010). In contrast to WT mice, IRF8-KO mice were unable to produce IFN- $\lambda$  to poly IC challenge in vivo, strongly suggesting that CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs were the source of IFN- $\lambda$  (Fig. 3 D).

#### **Poly IC injection in vivo induces IFN- $\lambda$ production in spleen cells with the phenotype of CD8 $\alpha^+$ cDCs**

Because gene deletions of the aforementioned mice may affect several functions, we decided to confirm the conclusion obtained so far. For this, we injected poly IC i.v. into WT mice, dissected the spleen, and cultured the splenocytes in vitro. IFN- $\lambda$  was detected in the supernatant of cultured total spleen cells (Fig. 4, A–C). Separation of the splenocytes with a density centrifugation, a step used to enrich DCs in the light density fraction, revealed that most IFN- $\lambda$  was associated with these light density cells. (Fig. 4 A).

As an alternative approach to investigate the role of DCs in the IFN- $\lambda$  production to poly IC in vivo, we used mice transgenic for the diphtheria toxin (DT) receptor (DTR) under the control of the CD11c promoter (CD11c-DTR-tg; Jung et al., 2002). CD11c is highly expressed in cDCs and to a minor extent in pDCs, some lymphocytes, and certain macrophages of the spleen (Jung et al., 2002; Probst et al., 2005). Our initial unpublished experiments confirmed that splenocytes of DT-untreated CD11c-DTR-tg mice produced IFN- $\lambda$  upon poly IC injection in vivo. Next, we treated WT and CD11c-DTR-tg mice with DT to ablate DCs in vivo. After injection of poly IC in vivo, spleen cells of the DT-treated WT but not DT-treated CD11c-DTR-tg mice produced IFN- $\lambda$  (Fig. 4 B), suggesting a role of CD11c $^+$  cells, probably DCs, as the main source of IFN- $\lambda$ .

The previous two separation experiments indicated that among splenocytes of poly IC-injected mice, light density DCs that highly expressed CD11c were the major source of IFN- $\lambda$ . To rule out other cell types and get an insight into the subset of DCs involved, poly IC was injected in WT mice, and 1.5–2 h later, total spleen cells were separated with a set of antibodies and magnetic beads into positive and negative fractions. The IFN- $\lambda$  production separated with cells negative for CD3 (T cells), CD45R (B cells and pDCs), CD11b (macrophages and CD8 $^-$  cDCs), CD172a (macrophages, pDCs, and CD8 $^-$  cDCs), and NK1.1 (NK cells) but positive for CD11c (DCs) and CD8 $\alpha$  (CD8 $\alpha$  cDCs and CTLs). Thus, upon poly IC injection in vivo, the IFN- $\lambda$  production in splenocytes segregated with cells of light density and the phenotype CD3 $^-$ , CD45R $^-$ , NK1.1 $^-$ , CD11b $^-$ , CD172a $^-$ , CD11c $^+$ , and CD8 $\alpha^+$ . This phenotype exactly describes CD8 $\alpha^+$  cDCs and excludes other cell types of the spleen such as lymphocytes or macrophages as major producers of IFN- $\lambda$  to poly IC injection in vivo.

#### **CD8 $\alpha^+$ cDCs are the major producers of IFN- $\lambda$ in response to poly IC in vitro**

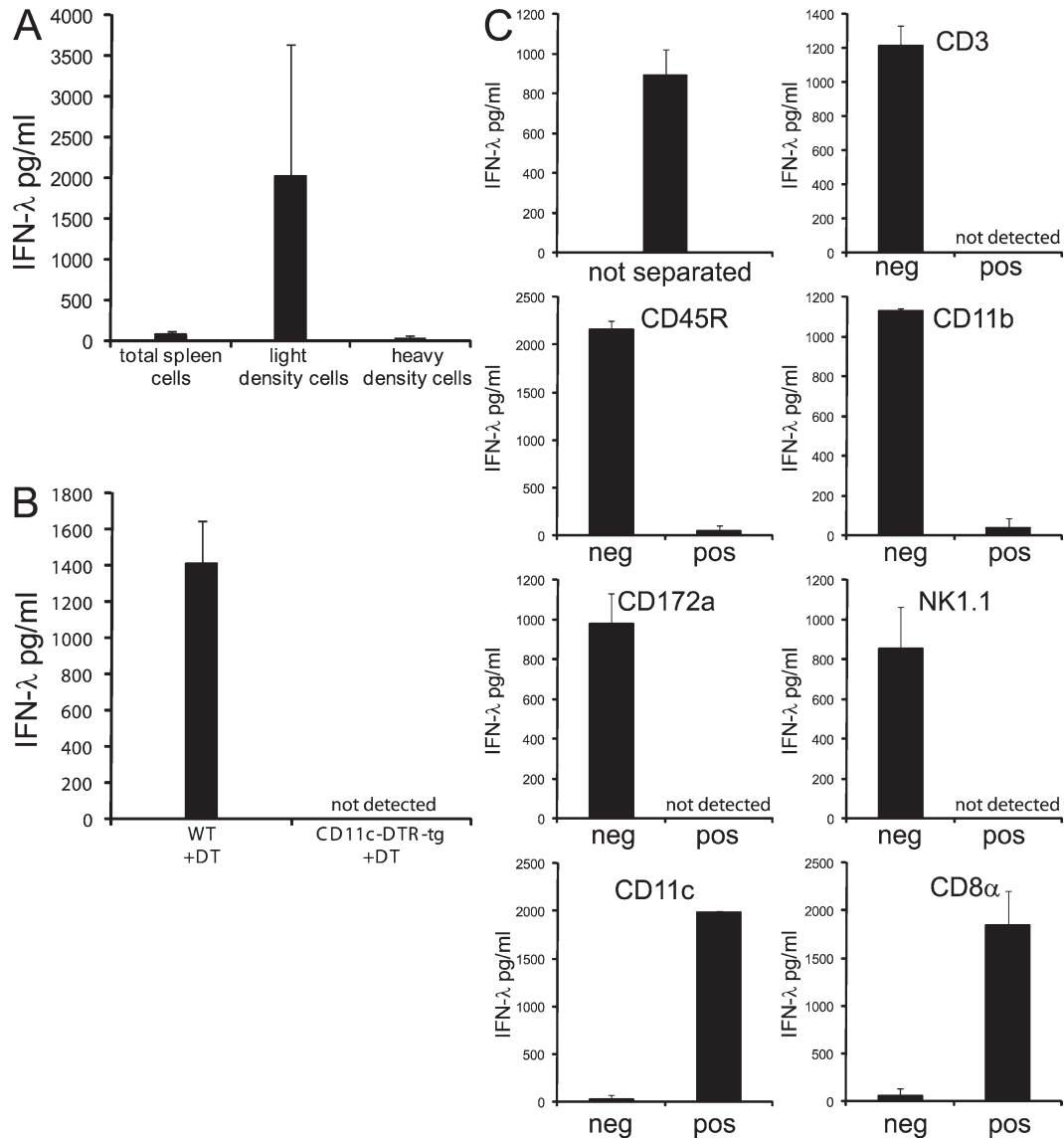
Stimulation of fractionated spleen cells with different TLR ligands in vitro revealed that the major lymphocyte fractions consisting of T and B lymphocytes were unable to produce

IFN- $\lambda$ , and its production was confined to enriched preparations of DCs (unpublished data). Therefore, we used a DC isolation protocol, which included density enrichment, depletion of T cells, B cells, NK cells, and granulocytes, and multiparameter fluorescence-activated cell sorting. Those highly purified DC subsets were stimulated with a panel of TLR ligands in vitro and IFN- $\lambda$ , and other cytokines and chemokines were determined in the supernatants. pDCs were the major source of IFN- $\lambda$  in response to the A-type ODN CpG-2216 (Fig. S1), as previously suggested by messenger RNA analysis of human pDCs (Coccia et al., 2004). However, in response to poly IC stimulation, the CD8 $\alpha^+$  cDCs were the major producers of IFN- $\lambda$ , with CD8 $^-$  cDCs, either expressing CD4 (CD4 $^+$  cDCs) or not (DN cDCs), and pDCs being largely unable to participate in IFN- $\lambda$  production (Fig. 5 A and Fig. S1 A). The IFN- $\lambda$  production was selective for poly IC stimulation and for CD8 $\alpha^+$  cDCs even though many different TLR ligands induced robust activation in CD8 $\alpha^+$  and CD8 $\alpha^-$  cDCs, as seen by the induction of the other cytokines (Fig. 5 B). As described previously, some TLR ligands were selective for certain DC subsets, with CD8 $\alpha^+$  cDCs being unresponsive to the TLR7 stimulation via R848, but in contrast being the sole responder to the TLR11 ligand profilin (Fig. 5 B and Fig. S1 B; Edwards et al., 2003; Yarovinsky et al., 2005).

#### **IFN- $\lambda$ and IL-12p70 production by CD8 $\alpha^+$ cDCs depends on the type of stimulus and the cytokine conditions**

CD8 $\alpha^+$  cDCs are well known for their exceptional capacity for IL-12p70 production. Because we found that the CD8 $\alpha^+$  cDCs were also able to produce large amounts of IFN- $\lambda$ , we next elucidated the conditions that would govern IFN- $\lambda$  compared with IL-12p70 production. By using a panel of different TLR stimuli, we found that TLR ligands known for their high IL-12p70 induction such as CpG-ODN or profilin of toxoplasma (Hochrein et al., 2000; Yarovinsky et al., 2005) induced large amounts of IL-12p70 as expected, but surprisingly the CD8 $\alpha^+$  cDCs did not produce any IFN- $\lambda$  under these conditions (Fig. 6 A). In contrast, poly IC stimulation induced IFN- $\lambda$  but not IL-12p70 production by CD8 $\alpha^+$  cDCs (Fig. 6 A). Combinations of poly IC together with Pam3Cys, LPS, CpG-ODN, or profilin, ligands for TLR2, TLR4, TLR9, or TLR11, respectively, synergistically increased IFN- $\lambda$  production (Fig. 6 A). The data demonstrate a synergistic increase of poly IC-induced IFN- $\lambda$  with MyD88-dependent stimuli and confirm described synergistic effects on the production of IL-12p70 by CD8 $\alpha^+$  cDCs (Fig. 6 A; Napolitani et al., 2005).

Previously, we and others have shown that the cytokine milieu during stimulation is highly influential for IL-12p70 production in murine and human DCs, with IL-4 being a major enhancer for bioactive IL-12 production (Hochrein et al., 2000; Kalinski et al., 2000). Using poly IC or CpG-1668 or a combination of poly IC and CpG-1668 as stimuli, we analyzed the effect of the addition of either IFN- $\gamma$  or IL-4 on the production of IFN- $\lambda$  and IL-12p70. IFN- $\gamma$  enhanced the production of IFN- $\lambda$  to poly IC or the combination of poly



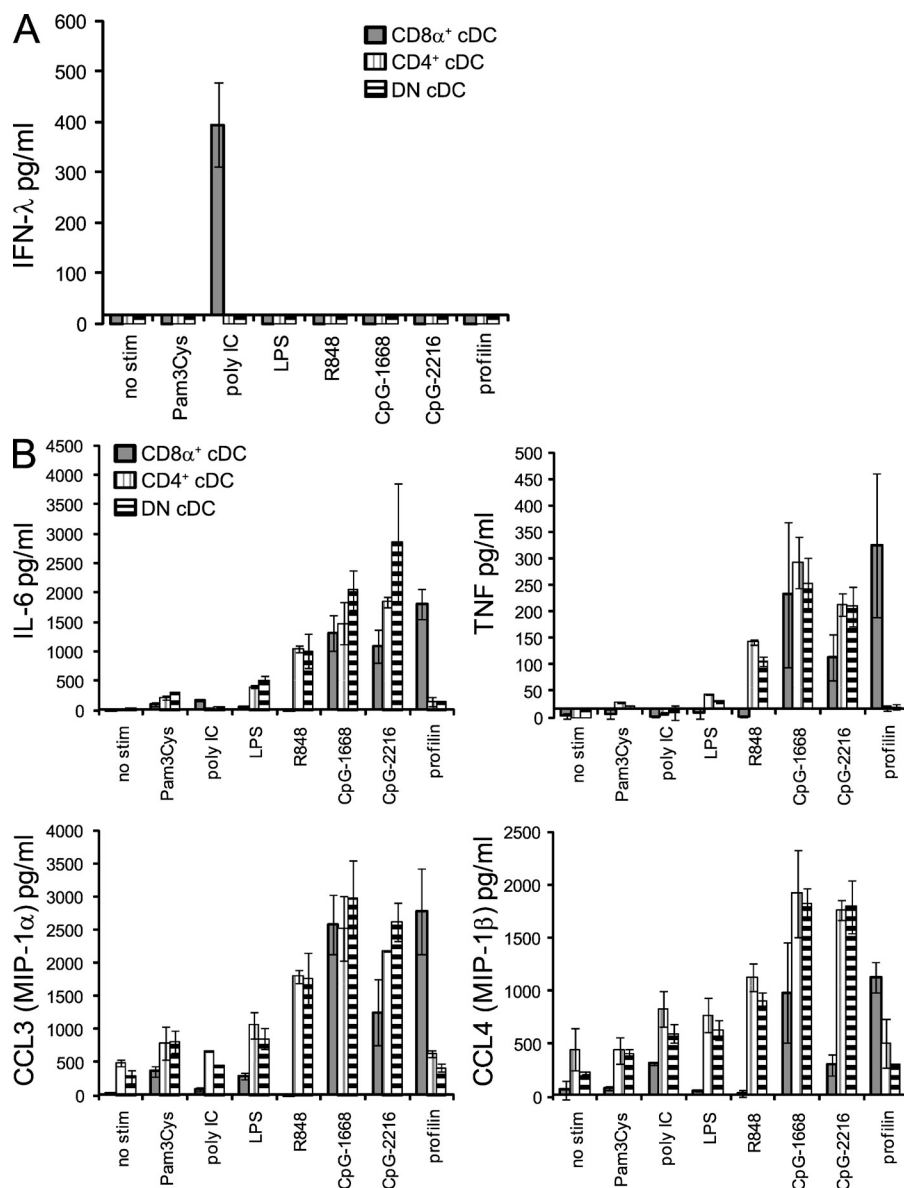
**Figure 4. The IFN- $\lambda$  production to poly IC injection in vivo separates with CD45R<sup>-</sup>/CD11c<sup>+</sup>/CD8 $\alpha$ <sup>+</sup> splenocytes.** 1.5–2 h after i.v. injection of poly IC, spleens were harvested and processed. Cell-free supernatants were analyzed for IFN- $\lambda$  after in vitro culture for 18 h. (A)  $5 \times 10^6$  cells/ml of total spleen cells or cells separated by density centrifugation into light density cells or heavy density cells. (B)  $25 \times 10^6$  cells/ml of total spleen cells of WT or CD11c-DTR-tg mice treated 2 d before with DT. (C) Total spleen cells before separation or after magnetic bead separation into the denoted populations. The initial cell number of splenocytes added onto the column was  $20 \times 10^6$ . Without further counting, each fraction was distributed into 2 wells with 200  $\mu$ l of medium/well. Bars represent the mean  $\pm$  SD of two independent experiments (A and C) or one experiment (B) using two mice per experiment.

IC and CpG-1668, whereas still no IFN- $\lambda$  was induced to CpG-1668 only (Fig. 6 B). As described previously, IFN- $\gamma$  had little effect on the IL-12p70 production, whereas IL-4 increased IL-12p70 but not IFN- $\lambda$  production (Fig. 6 B; Hochrein et al., 2000).

#### In vivo and in vitro FL-induced equivalents of CD8 $\alpha$ <sup>+</sup> cDCs produce IFN- $\lambda$ to poly IC

Spleen cDCs contain a very small fraction of DCs, which do not express CD8 $\alpha$  but otherwise resemble the CD8 $\alpha$ <sup>+</sup> cDCs in phenotype and their cross-presentation ability and are

thus regarded as eCD8 $\alpha$  cDCs (Bedoui et al., 2009). These cells are expanded by repeated FL injections. To test whether ex vivo isolated eCD8 $\alpha$  cDCs share other functional similarities with CD8 $\alpha$ <sup>+</sup> cDCs, we expanded DCs with FL in vivo and sorted CD8 $\alpha$ <sup>+</sup> cDCs, eCD8 $\alpha$  cDCs, and the remaining cDCs, which highly express CD11b and CD172a. Both, the CD8 $\alpha$ <sup>+</sup> and eCD8 $\alpha$  cDCs but not the CD11b/CD172a-expressing cDCs produced large amounts of IFN- $\lambda$  to poly IC and high IL-12p70 to CpG-1668 or profilin, indicating that eCD8 $\alpha$  cDCs share the high IFN- $\lambda$ - and IL-12p70-producing capacity of CD8 $\alpha$ <sup>+</sup> cDCs (Fig. 7 A).



**Figure 5. Splenic CD8 $\alpha^+$  cDCs are the major producers of IFN- $\lambda$  in response to poly IC in vitro.** Highly purified  $5 \times 10^5$  cells/ml of splenic cDC subsets were stimulated in the presence of IL-3 and GM-CSF with the stimuli as indicated. (A and B) After 18 h, supernatants were analyzed for IFN- $\lambda$  (A) or IL-6, TNF, CCL3, and CCL4 (B). Bars represent the mean  $\pm$  SD of three (A) or two (B) independent experiments using the pool of at least eight mice per experiment.

generated and sorted eCD8 $\alpha$  cDCs from the FL cultures of WT, MyD88-KO, Cardif-KO, TLR3-KO, and IFN-IR-KO mice. The production of IFN- $\lambda$  by FL-eCD8 $\alpha$  cDCs upon stimulation with poly IC was dependent on TLR3 and IFN-IR but was independent of MyD88 and Cardif (Fig. S3, A–D). Thus, the systemic production of IFN- $\lambda$  in vivo and the production in vitro by directly stimulated eCD8 $\alpha$  cDCs were governed by the same set of receptors, especially TLR3, which is highly expressed and known to be used for poly IC detection by CD8 $\alpha^+$  cDCs (Schulz et al., 2005). These data strongly suggest that CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs are the source of poly IC-induced IFN- $\lambda$  in vivo.

#### Production of IFN- $\lambda$ and IL-12p70 upon selective stimulation indicates the presence of CD8 $\alpha^+$ or eCD8 $\alpha$ cDCs in the liver

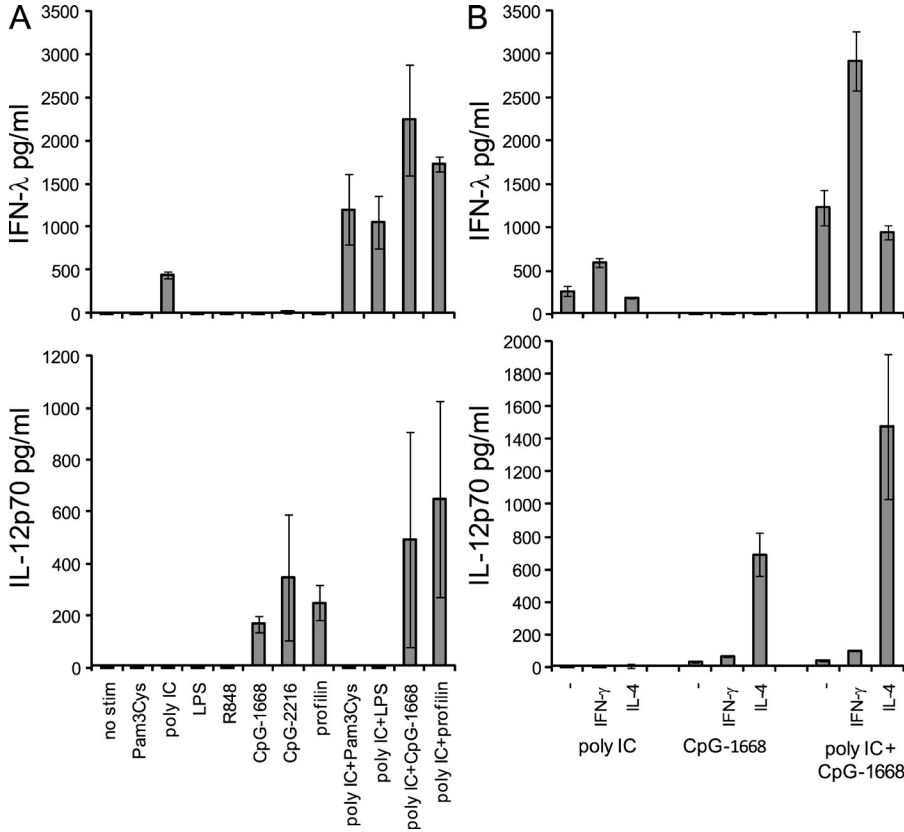
Based on the data accumulated thus far, we hypothesized that the production of IFN- $\lambda$  and IL-12p70 exclusively induced by certain stimuli in

The culture of BM cells with FL in vitro induces the development of DC subsets, including eCD8 $\alpha$  cDCs, with phenotypic, developmental, and functional similarities with CD8 $\alpha^+$  cDCs (Hildner et al., 2008; Naik et al., 2010). Comparing eCD8 $\alpha$  cDCs and CD11b/CD172a-expressing cDCs revealed that similar to ex vivo isolated cDC subsets, the mutually exclusive stimulus-dependent production of IFN- $\lambda$  or IL-12p70 was mostly confined to the eCD8 $\alpha$  subset (Fig. 7 B). Thus, eCD8 $\alpha$  cDCs either ex vivo isolated or in vitro generated share with CD8 $\alpha^+$  cDCs the ability for high IFN- $\lambda$  as well as IL-12p70 production.

In vivo, we had found that poly IC-induced IFN- $\lambda$  was independent of the presence of the adaptor molecules MyD88 and Cardif but depended on TLR3 and IFN-IR (Fig. 1). Because the in vitro generated eCD8 $\alpha$  cDCs showed such a similar production of IFN- $\lambda$  as their ex vivo isolated counterparts, we

CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs might be used to identify the presence of these DCs in complex mixtures of cells. For the highest production of both factors in vitro, we used a combination of cytokines (IL-3 + GM-CSF + IL-4 + IFN- $\gamma$ ), which promotes both IFN- $\lambda$  and IL-12p70 production. As stimuli, we used poly IC or proflin or the combination of poly IC + proflin, which are stimuli highly selective for CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs (Fig. 5 and Fig. S1). First, we wanted to test the selectivity of these stimuli for CD8 $\alpha^+$  cDCs within splenic cDC subsets. Indeed, we confirmed the stimulus-dependent production of IFN- $\lambda$  to poly IC, IL-12p70 to proflin, or the combined production of IFN- $\lambda$  and IL-12p70 to the combination of poly IC + proflin exclusively by CD8 $\alpha^+$  cDCs (Fig. S2 A). Stimulations of nonparenchymal liver cells under these conditions revealed the same pattern of IFN- $\lambda$  and IL-12p70 production (Fig. S2 B), indicating the presence of



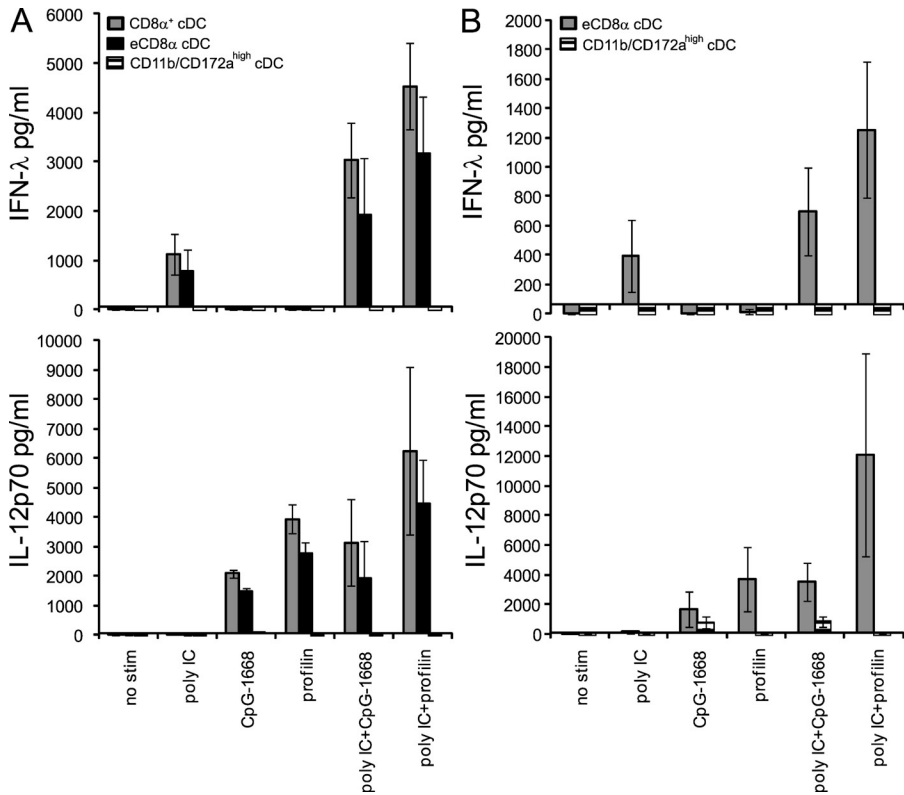


**Figure 6. The production of IFN-λ or IL-12p70 by CD8α<sup>+</sup> cDCs depends on the stimuli and the cytokine conditions.** 5 × 10<sup>5</sup> cells/ml of sorted splenic CD8α<sup>+</sup> cDCs were stimulated, and supernatants were analyzed after 18 h for IFN-λ and IL-12p70. (A) Stimulation in the presence of IL-3 and GM-CSF with the stimuli as indicated. (B) Stimuli and cytokines as indicated. Bars represent the mean ± SD of two independent experiments using a pool of at least eight mice per experiment.

CD8α<sup>+</sup> or eCD8α cDCs as previously shown (Sumpter et al., 2007). Liver cells from FL-KO mice, known to have drastically reduced numbers of DCs, were largely unable to produce IFN-λ or IL-12p70 under these conditions (Fig. S2 B).

**CD8α<sup>+</sup> cDCs, eCD8α cDCs, and pDCs produce IFN-λ to HSV-1 and parapoxvirus**

To extend our analysis of IFN-λ production in response to the dsRNA viral mimic poly IC to bona fide viruses, we stimulated ex vivo isolated splenic DC subsets from FL-expanded mice and DC subsets of in vitro generated FLDCs with HSV-1, parapoxvirus, and Sendai virus and analyzed the supernatants for IFN-λ. Among the spleen DC subsets, CD8α<sup>+</sup> cDCs, eCD8α cDCs, and pDCs produced large amounts of IFN-λ in response to HSV-1 and parapoxvirus (Fig. 8 A). The CD8<sup>-</sup> cDCs sorted as CD11b/CD172a<sup>high</sup> cDCs produced only very limited but, in contrast to the stimulation with poly IC, detectable amounts (between 100 and 400 pg/ml) of IFN-λ upon stimulation with HSV-1 or parapoxvirus.

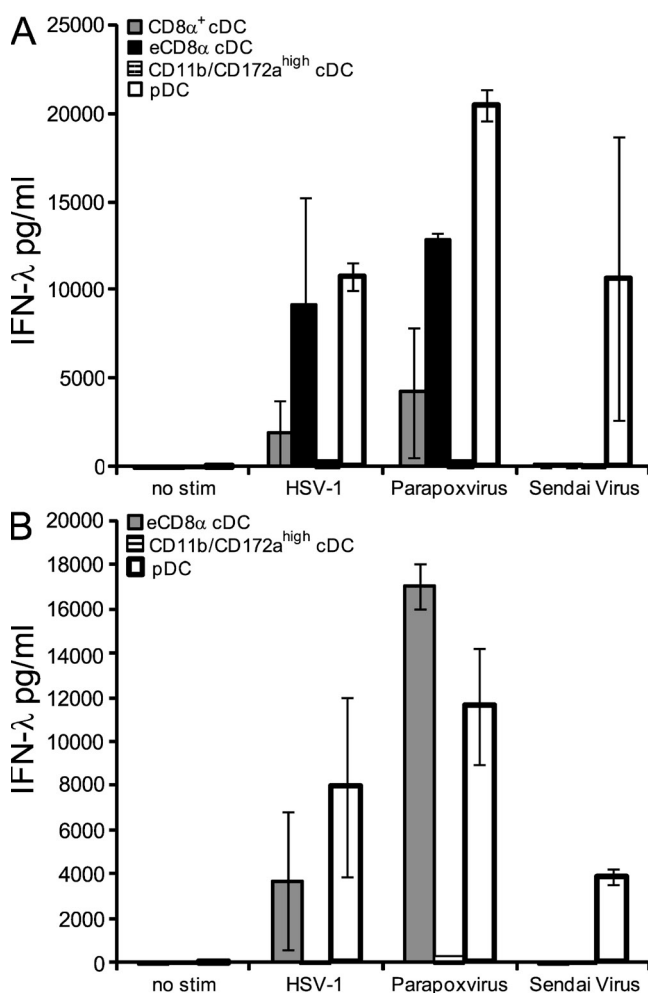


**Figure 7. In vivo and in vitro FL-generated CD8α<sup>+</sup> cDCs and eCD8α cDCs are major producers of IFN-λ and IL-12p70.** (A and B) 5 × 10<sup>5</sup> cells/ml of highly purified splenic DCs from FL-treated mice (A) or FLDCs (B) were stimulated in the presence of IL-3 + GM-CSF + IL-4 + IFN-γ with the stimuli as indicated. After 18 h, supernatants were analyzed for IFN-λ and IL-12p70. Bars represent the mean ± SD of two independent experiments each using a pool of at least two mice per experiment.

Only pDCs and none of the cDC subsets produced IFN- $\lambda$  in response to Sendai virus (Fig. 8 A). FLDC subsets generated in vitro displayed a very similar pattern with IFN- $\lambda$  production to HSV-1 and parapoxvirus mostly associated with eCD8 $\alpha$  cDCs and pDCs with a very limited contribution by CD11b/CD172a<sup>high</sup> cDCs (Fig. 8 B). Again, all IFN- $\lambda$  to Sendai virus was selectively produced by pDCs but not from the cDC subsets (Fig. 8 B). Thus, like pDCs, CD8 $\alpha$ <sup>+</sup> and eCD8 $\alpha$  cDCs are able to respond in vitro to some viruses with the production of large amounts of IFN- $\lambda$ .

### HSV-1 injection in vivo indicates that among splenocytes, pDCs and CD8 $\alpha$ <sup>+</sup> cDCs produce IFN- $\lambda$

Similar to our approach to identify the IFN- $\lambda$  producers among spleen cells in response to poly IC injection (Fig. 4 C),



**Figure 8.** In vivo and in vitro FL-generated CD8 $\alpha$ <sup>+</sup> cDCs, eCD8 $\alpha$  cDCs, and pDCs are major producers of IFN- $\lambda$  to HSV-1 and parapoxvirus. (A and B)  $5 \times 10^5$  cells/ml of highly purified splenic DCs from FL-treated mice (A) or FLDCs (B) were stimulated in the presence of IL-3 + GM-CSF + IL-4 + IFN- $\gamma$  with the stimuli as indicated. After 18 h, supernatants were analyzed for IFN- $\lambda$ . Bars represent the mean  $\pm$  SD of two independent experiments each using a pool of at least two mice per experiment.

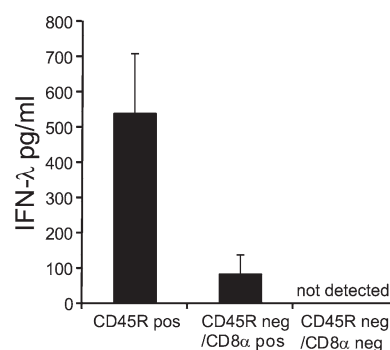
we performed experiments injecting HSV-1. In contrast to the results with poly IC, the majority of the HSV-1-induced IFN- $\lambda$  was within the CD45R-positive fraction (Fig. 9). This suggested that probably pDCs, which beside B cells express CD45R, are the major producers. We separated the CD45R-negative fraction into a CD8 $\alpha$ -positive and -negative fraction of cells. All remaining IFN- $\lambda$  production within the CD45R-negative fraction segregated with the CD8 $\alpha$ -positive cells (Fig. 9). These data suggest that the CD8 $\alpha$ <sup>+</sup> cDCs, which are CD45R<sup>-</sup>/CD8 $\alpha$ <sup>+</sup>, participate in the IFN- $\lambda$  production upon HSV-1 inoculation in vivo.

### Human BDCA3<sup>+</sup> DCs are the major producers of IFN- $\lambda$ s upon poly IC stimulation

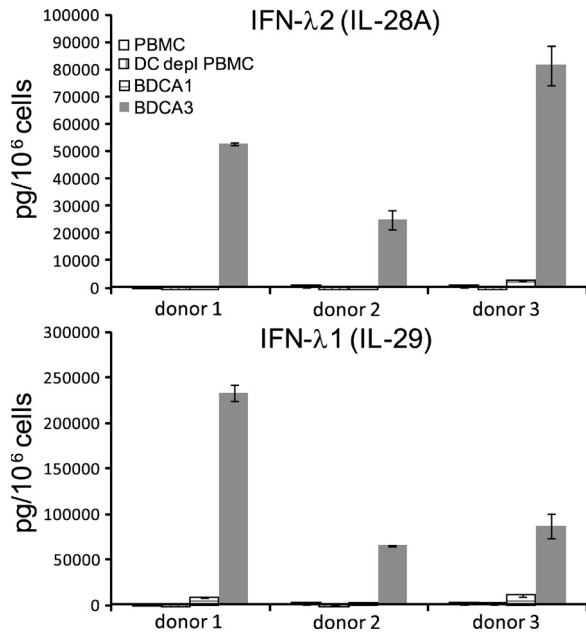
We found that the IFN- $\lambda$  production in response to poly IC in mice is a CD8 $\alpha$ <sup>+</sup> cDC subset-specific feature, and therefore, we wanted to determine whether this function correlated with any human DC subset. Given that human BDCA3<sup>+</sup> DCs are attributed as functional correlates of CD8 $\alpha$ <sup>+</sup> cDCs, we stimulated PBMCs, PBMCs depleted of cDCs, and highly enriched fractions of BDCA1<sup>+</sup> and BDCA3<sup>+</sup> cDCs and measured IFN- $\lambda$ 1 and IFN- $\lambda$ 2 in the supernatants. In PBMCs (Fig. 10) and fractions of DC-enriched PBMCs (not depicted), we found that poly IC induced IFN- $\lambda$ 1 (IL-29) and IFN- $\lambda$ 2 (IL-28A). Separation of cDC subsets using the markers BDCA1 or BDCA3 revealed that the BDCA3<sup>+</sup> cells were the major producers of IFN- $\lambda$ 1 as well as IFN- $\lambda$ 2 (Fig. 10). Thus, IFN- $\lambda$  production upon poly IC stimulation is another functional attribute that the human BDCA3<sup>+</sup> cDCs share with murine CD8 $\alpha$ <sup>+</sup> and eCD8 $\alpha$  cDCs.

## DISCUSSION

In this study, we have shown that poly IC, an outstanding adjuvant, induces IFN- $\lambda$  production by human BDCA3<sup>+</sup> (CD141<sup>+</sup>) and mouse CD8 $\alpha$ <sup>+</sup> cDCs, subsets which are also



**Figure 9.** The IFN- $\lambda$  production to HSV-1 injection in vivo separates with CD45R<sup>+</sup> and CD45R<sup>-</sup>/CD8 $\alpha$ <sup>+</sup> splenocytes. Spleen cells 1.5 h after in vivo injection with DISC-HSV-1 were separated with anti-CD45R and magnetic beads into positive and negative fractions. The CD45R-negative fraction was further separated into cells positive or negative for CD8 $\alpha$ . Separated cells were cultured in vitro for the next 18 h, and cell-free supernatants were analyzed for IFN- $\lambda$ . Bars represent the mean  $\pm$  SD of two independent experiments using one mouse per experiment.



**Figure 10. Human BDCA3<sup>+</sup> cDCs are major producers of IFN-λ upon poly IC stimulation.** PBMCs, PBMCs depleted of BDCA1 and BDCA3, or cells positively selected either for BDCA1 or BDCA3 were stimulated in the presence of IL-3, GM-CSF, and IFN-γ with 100 μg/ml poly IC + 10 μg/ml Pam3Cys + 10 μg/ml LPS (donor 1) or with 100 μg/ml poly IC for 18–24 h (donors 2 and 3). Supernatants were analyzed for IFN-λ1 and IFN-λ2. The experiments are shown for the individual donors, and data represent mean ± SD of duplicate samples.

specialized in IL-12p70 production and cross-presentation. In response to poly IC, IFN-λ was not coexpressed with IL-12p70 unless MyD88-dependent stimuli were also sensed by these cells. Because poly IC is shown in this study to directly target CD8α<sup>+</sup> cDCs for IFN-λ induction, it is tempting to speculate that this cytokine constitutes an important component of the poly IC-induced adjuvant response. Understanding the individual adjuvant components of poly IC may permit the design of vaccines with enhanced antiviral responses but minimal side effects. Moreover, poly IC is a mimic of viral dsRNA, and the IFN-λ responses to this adjuvant are potentially mirrored in the response to numerous DNA and RNA viruses that produce dsRNA intermediates or potentially other pathogen-associated patterns. The recent studies linking IFN-λ to hepatitis C viral clearance highlight the potential importance of this cytokine in viral disease (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009). Moreover, the role of IFN-λ extends beyond viral disease. It is reported that T<sub>H</sub>1-inducing properties (Jordan et al., 2007) could have potential use as an adjuvant in airway diseases in which its production is possibly deficient (Contoli et al., 2006). It has also been shown that IFN-λ induces apoptosis in epithelial cell tumors (Brand et al., 2005), raising the possibility that it may function as a tumor suppressor.

Poly IC is a well-known agent that induces systemic levels of IFN-α in experimental animals. Until recently, the molecular

and cellular bases for this IFN-α were unknown. We and others have described that CD8α<sup>+</sup> cDCs but not the CD8<sup>-</sup> cDCs were producers of IFN-α in response to poly IC stimulation in vitro (Hochrein et al., 2001; Longhi et al., 2009). Recently, it became clear that DCs are not responsible for the systemic production of IFN-α. Poly IC-driven IFN-α induction depends on the RLH MDA5 and originates from cells of non-hematopoietic origin (Gitlin et al., 2006; Longhi et al., 2009). These data were further corroborated in TLR3-KO and Batf3-KO mice, which lack the dsRNA recognition receptor predominantly used by CD8α<sup>+</sup> cDCs or CD8α<sup>+</sup> cDCs per se, respectively. Induction of poly IC-induced systemic IFN-α was not impaired in these mice (Gitlin et al., 2006; Longhi et al., 2009; McCartney et al., 2009; Edelson et al., 2010).

In this study, we have provided evidence that poly IC induces early systemic IFN-λ production. To our knowledge, this is the first report of directly measured systemic IFN-λ in response to poly IC in vivo. Indirect evidence of systemic poly IC-induced IFN-λ was provided by Ank et al. (2008), who nicely demonstrated that antiviral protection induced by poly IC partially depended on the presence of the IFN-λ receptor (IL-28R).

In vitro, the induction of IFN-λ transcripts was reported by the first papers that described the discovery of the IFN-λs (Kotenko et al., 2003; Sheppard et al., 2003). Many studies have since extended those findings in several cell types in vitro, including GM-CSF-derived murine and human monocyte-derived DCs (GM-DCs), macrophages, and pDCs (for review see Ank and Paludan, 2009). One study demonstrated induction of IFN-λ upon stimulation with Sendai virus, HSV-2, poly IC, and B-type ODN (CpG-1826) in GM-DCs (Ank et al., 2008) and another with LPS or CD40L (Wolk et al., 2008). In contrast, we could only find limited amounts of IFN-λ in ex vivo isolated or in vitro with FL-generated CD11b/CD172a<sup>high</sup> cDCs induced by the DNA viruses HSV-1 and parapoxvirus but none to Sendai virus (Fig. 8), to poly IC, or the other TLR ligands (Figs. 5 and 7), even though the viruses induced some IFN-α in those CD11b<sup>high</sup> cDCs (Hochrein et al., 2004; Siegemund et al., 2009; Lubber et al., 2010). We have also analyzed GM-DCs, but again we could only find limited amounts of IFN-λ to HSV and parapoxvirus but none to Sendai virus or poly IC or a panel of TLR ligands including B-type ODN (CpG-1668; unpublished data). However, comparing the protocols for the generation of GM-DCs, we realized substantial differences in the duration of culture and thus the maturation state of these DCs. It will be interesting to elucidate the basis of the differences in the IFN-λ production capacity of non-CD8α<sup>+</sup> cDCs. It is important to note that our in vivo analyses and ex vivo isolated DC subset analyses and organ stimulations were performed using healthy pathogen-free mice. Under these noninflammatory steady-state conditions, all of our in vivo and in vitro data point to CD8α<sup>+</sup> and eCD8α cDCs as the sole and nonredundant producers of early IFN-λ upon poly IC exposure. It has been shown previously that these steady-state conditions have no substantial numbers of inflammatory DCs (e.g., monocyte-derived DCs

or TIP DCs; for review see Shortman and Naik, 2007). This situation may change dramatically upon infection or inflammation, circumstances which we have not analyzed. It is known that inflammatory cytokines such as IFN- $\beta$ , IFN- $\gamma$ , GM-CSF, or TNF dramatically alter the cytokine production capacity of many immune and nonimmune cells, and some studies indicate that this might also be the case for the production of IFN- $\lambda$ s (Sirén et al., 2005; Ank et al., 2006; Megjugorac et al., 2010).

We found that systemic IFN- $\lambda$  originated from hematopoietic cells and among those exclusively from CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs, and its production was fully dependent on TLR3 but independent of the adaptor molecules for other TLRs or RLHs, MyD88 or Cardif, respectively. In contrast, and confirming previous data (Gitlin et al., 2006; Longhi et al., 2009), we found that the production of systemic IFN- $\alpha$  was independent of TLR3 but fully dependent on Cardif. CD8 $\alpha^+$  cDCs highly express and recognize poly IC via TLR3 (Edwards et al., 2003; Schulz et al., 2005), whereas they hardly express the RLHs (Luber et al., 2010). It is interesting to see that the same danger signal (dsRNA) induces early and high systemic levels of two different IFN families. This danger signal is not only sensed by two different families of PRRs (TLRs and RLHs) but also by completely different cellular sources (CD8 $\alpha^+$  cDCs and nonhematopoietic cells).

Mice deficient in FL have drastically reduced but not fully absent numbers of DCs, including CD8 $\alpha^+$  cDCs. Based on the reduced numbers of DCs, we expected to detect residual IFN- $\lambda$  production upon poly IC stimulation. However, the production of IFN- $\lambda$  by FL-KO mice was nearly completely abolished in vivo (Fig. 3 C) and in liver cells in vitro (Fig. S2 B). Previously, we found that pDCs isolated from FL-KO mice were inhibited in their production of IFN- $\alpha$  upon TLR9 stimulation (unpublished data). This suggested that DCs from FL-deficient mice might also be functionally impaired or alternatively that these non-FL-dependent DCs are a subset of the normal steady-state DC populations and do not share the same functional attributes with FL-dependent DCs. In line with our observations, it was found recently that mice with a mutation in the receptor for FL (flt3) demonstrated a severely reduced production of cytokines upon TLR stimulation of their DCs, including CD8 $\alpha^+$  cDCs (Eidenschenk et al., 2010).

Mice deficient for IRF8 have defects in the development of pDCs and CD8 $\alpha^+$  and eCD8 $\alpha$  cDCs (Aliberti et al., 2003; Edelson et al., 2010). In line with the absence of CD8 $\alpha^+$  cDCs, we found that IRF8-KO mice were unable to produce systemic IFN- $\lambda$  to poly IC. Like FL, IRF8 seems to play an additional functional role, as demonstrated by the impaired IFN- $\alpha$  production by pDCs (Tailor et al., 2007), which was not further examined in our study. However, it is interesting to note that both DC subsets able to produce large amounts of IFN- $\lambda$ , pDCs and CD8 $\alpha^+$  cDCs, not only depend on IRF8 for their development but also express high levels of IRF8 when isolated as end-stage cells from spleen (Luber et al., 2010). Whether the highly expressed IRF8 of these two professional

IFN- $\lambda$ -producing DC subsets is somehow linked to the IFN- $\lambda$  producing capacity grants further examination.

In this study, we describe that IRF3 and IRF7 play important roles in the poly IC-induced IFN- $\lambda$  and IFN- $\alpha$  production in vivo (Fig. 2). A role for IRF3 has been previously described for the induction of IFN-I downstream of both PRRs involved, the RLHs as well as for TLR3 (Tamura et al., 2008). Interestingly, in the absence of IRF3, no IFN- $\lambda$  but some remaining IFN- $\alpha$  was produced, whereas in the absence of IRF7, we found a remaining IFN- $\lambda$  but completely abrogated IFN- $\alpha$  production. An essential role for IRF7 has been demonstrated previously for MyD88-dependent IFN- $\alpha$  production by pDCs, and a participation of IRF7 in TRIF-dependent IFN-I production by DCs has been proposed (Honda et al., 2005; Tamura et al., 2008). Our in vivo findings of a prominent role for both IRF3 and IRF7 for the production of IFN- $\lambda$  in response to poly IC are in line with previous promoter-based data (Osterlund et al., 2007). We have performed some preliminary experiments with eCD8 $\alpha$  cDCs sorted from FLDCs, which confirmed the complete dependence on IRF3 for the production of IFN- $\lambda$  in response to poly IC (unpublished data). For stimulations of eCD8 $\alpha$  cDCs from IRF7-KO with poly IC, the role of IRF7 seemed to be less prominent, which makes it possible that the role of IRF7 in vivo is indirect, for example via the complete absence of IFN- $\alpha$  (Fig. 2), but this has to be confirmed with more detailed experiments. We have shown that the IFN- $\lambda$  production in IFN-IR-deficient mice is not only reduced upon poly IC in vivo (Fig. 1) but also by eCD8 $\alpha$  cDCs stimulated with poly IC in vitro (Fig. S3 D), suggesting that the lack of IFN-IR on CD8 $\alpha^+$  cDCs is directly responsible for this effect. The expression of IFN-IR by DCs and especially by CD8 $\alpha^+$  cDCs was previously shown to be important for their function in antigen presentation (Longhi et al., 2009; Cucak et al., 2009), and a positive feedback mechanism by IFN-I on the production of IFN- $\lambda$  has previously been described (Ank et al., 2008). Because the IFN- $\lambda$  production to poly IC in vivo and in vitro was not fully abrogated in the absence of the IFN-IR, this leaves a window for the activity of poly IC-induced IFN- $\lambda$  in the absence of the IFN-IR. Indeed, Ank et al. (2008) have shown that the protective effect of poly IC to HSV infection was partially dependent on the IFN- $\lambda$ R and functional in the absence of IFN-IR. However, IRF8 is highly expressed by CD8 $\alpha^+$  DCs (Luber et al., 2010), and both IRF3 and IRF7 play a role in IFN- $\lambda$  production by CD8 $\alpha^+$  DC in response to poly IC. It is possible that the role of IFN-IR is not only limited to an IFN-I feedback signaling at the time of stimulation but that it might play a role in the expression and/or regulation of essential IRFs such as IRF8, IRF3, or IRF7 in the DCs during their development. This is currently under investigation.

We found that upon poly IC injection in vivo, only splenic CD11c $^+$ /CD8 $\alpha^+$  cells but not other splenic cell populations such as lymphocytes or macrophages could produce IFN- $\lambda$ . Highly purified splenic DCs stimulated in vitro confirmed that the CD8 $\alpha^+$  cDCs selectively produced IFN- $\lambda$  to poly IC but not to any other sole TLR ligand, whereas pDCs produced



IFN- $\lambda$  to CpG-2216. Beside ex vivo isolated CD8 $\alpha^+$  cDCs, equivalents of those cells (eCD8 $\alpha$  cDCs) expanded by FL in vivo or in vitro shared the high IFN- $\lambda$ - and IL-12p70-producing capacity, a feature previously not described for ex vivo isolated FL-expanded eCD8 $\alpha$  cDCs. eCD8 $\alpha$  cDCs have been identified based on their dependence on the transcription factors IRF8 or Batf3 to be present in several lymphoid and nonlymphoid organs (Hildner et al., 2008; Edelson et al., 2010). Our selective stimulations of liver cells indicated that eCD8 $\alpha$  or CD8 $\alpha^+$  cDCs were present, and cells with this phenotype have been previously identified in the liver (Sumpter et al., 2007). Thus, IFN- $\lambda$  production seems to be a function of lymphoid and nonlymphoid organ eCD8 $\alpha$  or CD8 $\alpha^+$  cDCs.

Research on human cDC subsets has, until recently, relied on phenotypical analysis, which indicated that certain surface markers such as Clec9a or Necl2 selectively expressed by mouse CD8 $\alpha^+$  cDCs were associated with a human blood DC subset that could be identified by expression of CD141 (BDCA3). We had previously shown that the IL-12p70 production of thymic human cDC subsets was preferentially localized to the CD11b $^-$  subset (Vandenabeele et al., 2001). Recently, the evidence for human BDCA3 $^+$  cDCs as the mouse eCD8 $\alpha$  equivalents was substantiated in parallel by four groups who demonstrated that among human cDCs, BDCA3 $^+$  cDCs, like their mouse counterparts, highly express TLR3, produce IL-12p70, and possess superior cross-presenting activity (Bachem et al., 2010; Crozat et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010; Villadangos and Shortman, 2010). We have now shown that the newly discovered function of mouse CD8 $\alpha^+$  and eCD8 $\alpha$  as the major producers of IFN- $\lambda$  in response to poly IC is conserved throughout evolution to the human eCD8 $\alpha$  (BDCA3 $^+$ ) cDCs.

We do not yet know all of the consequences of the dedication of a single cell type to produce IFN- $\lambda$  to dsRNA, nor do we know the full consequences of IFN- $\lambda$  in the network of defense and immunoregulation. However, the strong functional conservation of similar cell types between mice and man is an indication of importance, and it strengthens the likelihood that certain aspects of IFN- $\lambda$  biology, including its regulation and novel therapeutic strategies based on experimental work performed in mice, will translate into humans.

## MATERIALS AND METHODS

**Mice.** Mice were bred and maintained either in the animal facilities at Bavarian Nordic GmbH, the Max Planck Institute of Biochemistry, the Max Planck Institute of Immunobiology, the Amrep Animal facility, the Walter and Eliza Hall Institute of Medical Research, the University of Melbourne, or at the University of Zurich according to institutional guidelines. Breeding stocks were originally obtained from MyD88-KO and TRIF-KO mice from S. Akira (Osaka University, Suita, Osaka, Japan; Adachi et al., 1998), Cardif-KO mice from J. Tschopp (University of Lausanne, Epalinges, Switzerland; Meylan et al., 2005), FL-KO mice (McKenna et al., 2000), transgenic mice that express the primate DTR under the control of the CD11c promoter from S. Jung (The Weizmann Institute of Science, Rehovot, Israel; Jung et al., 2002), TLR3-KO (Alexopoulou et al., 2001), IL-15R-KO (Lodolce et al., 1998) and RAG1-KO (Mombaerts et al., 1992) mice from The Jackson Laboratory, IRF3-KO and IRF7-KO mice from T. Taniguchi (University of Tokyo,

Bunkyo-ku, Tokyo, Japan; Honda et al., 2005), IRF8-KO from I. Horak (Leibniz Institut für Molekulare Pharmakologie, Berlin, Germany; Holtschke et al., 1996), and C57BL/6 WT mice purchased from Harlan Winkelmann GmbH, Eleavage Janvier, or the Walter and Eliza Hall Institute of Medical Research bred C57BL/6. Protocols for animal experimentation were approved by the government of upper Bavaria (Regierung von Oberbayern) or by the Walter and Eliza Hall Institute of Medical Research or University of Melbourne Animal Ethics Committees.

**Cells and flow cytometric sorting.** DC subsets were isolated from pooled mouse spleens as described previously (Vremec et al., 2007). In brief, spleens were chopped, digested with collagenase (Worthington Biochemical) and DNase (Roche) at room temperature, and treated with EDTA. Low-density cells were enriched by density centrifugation; non-DC lineage cells were coated with mAbs (anti-CD3 [KT3-1.1], anti-Thy-1 [T24/31.7], anti-Gr-1 [1A8], anti-CD19 [1D3], anti-erythrocytes [TER119], and anti-NK cells [DX5]) and depleted using anti-rat Ig magnetic beads (QIAGEN). Dead cells were excluded by propidium iodide staining. cDC populations were sorted based on the expression of CD11c, CD45RA, CD4, CD8 $\alpha$ , and CD172a, and pDCs were purified based on CD11c, CD45RA, and CD172a (all from BD) expression. Cell sorting was performed on a FACS Aria instrument (BD).

To generate FL-induced DCs in vivo, mice were treated i.p. with nine doses of FL (10  $\mu$ g/day), and spleen DCs were enriched on day 10 as described in the previous paragraph. pDCs were sorted as CD11c $^{int}$ , CD45RA $^{high}$ , CD11b $^{neg}$ , and CD172a $^{int}$  cells; CD8 $\alpha^+$  cDCs were sorted as CD11c $^{pos}$ , CD45RA $^{neg}$ , CD11b $^{neg/low}$ , CD172a $^{neg}$ , CD24 $^{high}$ , and CD8 $\alpha^{pos}$  cells; eCD8 $\alpha$  cDCs were sorted as CD11c $^{pos}$ , CD45RA $^{neg}$ , CD11b $^{neg/low}$ , CD172a $^{neg}$ , CD24 $^{high}$ , and CD8 $\alpha^{neg}$  cells; CD11b/CD172a $^{high}$  cDCs were sorted as CD11c $^{pos}$ , CD45RA $^{neg}$ , CD11b $^{high}$ , CD172a $^{high}$ , CD24 $^{neg/low}$ , and CD8 $\alpha^{neg}$  cells.

FL BM culture-derived DCs (FLDCs) were prepared as described previously (Hochrein et al., 2004). pDCs and eCD8 $\alpha$  and CD11b/CD172a $^{high}$  cDC subsets were sorted based on the expression of CD11c, CD45R, CD11b, CD24, and CD172a (all from BD).

**In vitro stimulation and cytokine detection.** Cells were stimulated in vitro with single TLR agonists or combinations thereof containing 10  $\mu$ g/ml Pam3Cys (InvivoGen), 100  $\mu$ g/ml poly IC (Axxora), 10  $\mu$ g/ml LPS (*Escherichia coli*; Sigma-Aldrich or Axxora), 10  $\mu$ g/ml R848 (Axxora), 1  $\mu$ M CpG-1668 or CpG-2216 (TIB-Molbiol), and 1  $\mu$ g/ml profilin of toxoplasma (Axxora). The recombinant cytokines mouse IL-3, mouse IL-4, rat IFN- $\gamma$  (PeproTech), and mouse GM-CSF (Tebu-Bio; 10 ng/ml each) were added as indicated. The addition of IL-3 and GM-CSF was based on our previous observations that GM-CSF promoted the production of IL-12p70 (Hochrein et al., 2000) and that the combination of IL-3 and GM-CSF increased virus-induced IFN- $\alpha$  production in pDCs and cDCs (Hochrein et al., 2004). DISC-HSV-1 (HSV-1 disabled infectious single cycle) is a replication-deficient form of HSV-1, which lacks the gene for glycoprotein H and has to be propagated on transgenic feeder cells. In noncomplementing cells, it can perform only a single cycle of infection (McLean et al., 1994). The virus has been shown to be equally potent as a WT HSV-1 to induce IFN- $\alpha$  in pDCs and cDCs in vitro (Hochrein et al., 2004). Inactivated parapoxvirus ovis used in veterinary science (Pfizer Animal Health) was described to induce IFN- $\alpha/\beta$  in pDCs and cDCs (Siegemund et al., 2009). Sendai virus strain Cantell (Charles River) was described to induce IFN- $\alpha$  in pDCs and CD11b $^{high}$  but not CD8 $\alpha^+$  cDCs (Luber et al., 2010). IFN- $\lambda$  in supernatants was analyzed by ELISA, and IL-12p70, IL-6, TNF, CCL3, and CCL4 were determined by FlowCytomix bead assay (Bender MedSystems) according to the manufacturer's protocol. Note that IFN- $\lambda$  was analyzed by an IFN- $\lambda$ 3 (IL-28B) ELISA (R&D Systems), but we found that this ELISA is largely cross-reactive to IFN- $\lambda$ 2 (IL-28A) and thus were not able to differentiate between these two mouse IFN- $\lambda$ s.

**Liver cell preparation.** To obtain liver cell suspensions for stimulation cultures, livers were harvested from mice, chopped into small pieces, and incubated with 1 ml collagenase D (1 mg/ml; Roche) at 37°C for 30 min. Single-cell suspensions were then prepared by mechanically disrupting the organs through



a 70- $\mu$ m filter. Nonparenchymal liver cells were further isolated by centrifugation in 35% Percoll (GE Healthcare) and then subjected to RBC lysis.

**In vivo challenge and magnetic bead separation.** Mice were injected i.v. into the lateral tail vein with 100  $\mu$ g poly IC (Axxora), and serum was collected 3–4 h after challenge. Sera were prediluted 1:5, and IFN- $\lambda$  (R&D Systems) and IFN- $\alpha$  (PBL) were analyzed by ELISA.

To separate splenocytes after i.v. poly IC or DISC-HSV-1 injection, spleens were dissected 1.5–2 h after challenge, erythrocytes were lysed (RBC lysing buffer; Sigma-Aldrich) and stained with the corresponding fluorochrome-labeled antibodies (against CD3, CD8 $\alpha$ , CD11b, CD11c, CD45R, CD172a, and NK1.1 [BD or eBioscience]), and the cells were separated with anti-fluorochrome magnetic beads as described by the manufacturer (Miltenyi Biotec) into a negative and positive enriched fraction. The initial cell number of splenocytes added onto an individual column per separation was  $20 \times 10^6$ . Without further counting or analyzing the purity, each fraction was distributed into 2 wells with 200  $\mu$ l of medium/well. For some experiments, the negative fraction was incubated with another round of beads using beads against a different fluorochrome as used for the first round of selection. All of the cells were incubated in vitro for the next 18 h, and cell-free supernatants were analyzed for IFN- $\lambda$  as described in In vitro stimulation and cytokine detection. For some experiments, the mice, either C57BL/6 WT or CD11c-DTR-tg, were treated with 100 ng DT (Sigma-Aldrich) i.p. 2 d before challenge with poly IC to deplete CD11c<sup>high</sup> transcribing cells.

**Isolation and stimulation of human DC.** PBMCs were prepared from peripheral blood of nonatopic blood donors by density gradient centrifugation, and BDCA3<sup>+</sup> DCs were purified from PBMCs using the BDCA3/CD141<sup>+</sup> DC isolation kit (Miltenyi Biotec) on an AutoMACS separator (Miltenyi Biotec). Subsequently, BDCA1<sup>+</sup> DCs were purified from the BDCA3-depleted PBMCs using the BDCA1/CD1c<sup>+</sup> DC isolation kit (Miltenyi Biotec). Preliminary experiments with PBMCs and DC-enriched fractions of PBMCs have indicated that the addition of the recombinant human cytokines IL-3, GM-CSF, and IFN- $\gamma$  (10 ng/ml each; all PeproTech) enhanced the IFN- $\lambda$ 1 and IFN- $\lambda$ 2 production, and accordingly, this combination of cytokines was added to all stimulations shown. After stimulation for 18–24 h, the supernatants were analyzed for IFN- $\lambda$ 1 and IFN- $\lambda$ 2 by ELISA according to the manufacturer's recommendations (Tebu-Bio).

**BM reconstitution.** Groups of three C57BL/6, TRIF-KO, or TLR3-KO mice were irradiated with  $2 \times 550$  rads, 2 h apart. They were rested for 3 h and then reconstituted by i.v. injection with  $5 \times 10^6$  red and dead cell-depleted BM cells of the required genotype. Mice were maintained on antibiotic-supplemented drinking water, and 4 wk after reconstitution, the mice were injected i.v. with 100  $\mu$ g poly IC. 4 h later, blood was taken for IFN- $\lambda$  assays.

**Statistical analysis.** Statistical significance was calculated using a two-tailed Student's *t* test. Results are expressed as means  $\pm$  SD.

**Online supplemental material.** Fig. S1 shows that splenic pDCs produce large amounts of IFN- $\lambda$  to CpG-2216. Fig. S2 shows that the production of IFN- $\lambda$  and IL-12p70 in response to specific stimulation suggests the presence of CD8 $\alpha$ <sup>+</sup> or eCD8 $\alpha$  cDCs in liver of WT but not FL-KO mice. Fig. S3 shows that IFN- $\lambda$  production to poly IC by FLDC-derived eCD8 $\alpha$  cDCs depends on TLR3 and IFN-IR but not on MyD88 or Cardif. Fig. S4 shows IFN- $\alpha$  production to poly IC in vivo by WT, IL-15R-KO, RAG-KO, and FL-KO mice. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092720/DC1>.

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