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Ulf Yrlid, Vuk Cerovic, Simon Milling, Christopher D. Jenkins, Jiquan Zhang, Paul R. Crocker, Linda S. Klavinskis and G. Gordon MacPherson

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# Plasmacytoid Dendritic Cells Do Not Migrate in Intestinal or Hepatic Lymph

Ulf Yrlid,\* Vuk Cerovic,<sup>†</sup> Simon Milling,\* Christopher D. Jenkins,\* Jiquan Zhang,<sup>‡</sup> Paul R. Crocker,<sup>‡</sup> Linda S. Klavinskis,<sup>†</sup> and G. Gordon MacPherson<sup>1\*</sup>

Plasmacytoid dendritic cells (pDCs) recognize pathogen-associated molecules, particularly viral, and represent an important mechanism in innate defense. They may however, also have roles in steady-state tolerogenic responses at mucosal sites. pDCs can be isolated from blood, mucosa, and lymph nodes (LNs). Although pDCs can express peripherally derived Ags in LNs and at mucosal sites, it is not clear whether pDCs actually migrate from the periphery in lymph or whether LN pDCs acquire Ags by other mechanisms. To determine whether pDCs migrate in lymph, intestine or liver-draining LNs were removed and thoracic duct leukocytes (TDLs) were collected. TDLs expressing MHC-II and CD45R, but not TCR $\alpha\beta$  or CD45RA, were then analyzed. These enriched TDLs neither transcribe type I IFNs nor secrete inflammatory cytokines in response to viral stimuli *in vitro* or after a TLR7/8 stimulus *in vivo*. In addition, these TDLs do not express CD5, CD90, CD200, or Siglec-H, but do express Ig, and therefore represent B cells, despite their lack of CD45RA expression. Intestinal and hepatic lymph are hence devoid of bona fide pDCs under both steady-state conditions and after TLR7/8 stimulation. This shows that any role for pDCs in Ag-specific T cell activation or tolerance must differ from the roles of classical dendritic cells, because it cannot result from peripheral Ag capture, followed by migration of pDCs via lymph to the LN. *The Journal of Immunology*, 2006, 177: 6115–6121.

It is now clear that plasmacytoid dendritic cells (pDCs)<sup>2</sup> play crucial roles in both innate defense and regulation of adaptive immune responses (1). Much, however, remains to be determined about the immunophysiology of these cells, particularly their migratory properties. pDCs were originally identified in human blood and tonsils. In mice, they have been characterized in bone marrow (2), blood (3), spleen (4, 5), lymph node (LN) (4, 5), liver (6), Peyer's patches (7), and mucosal tissues, e.g., lung (8). pDCs appear to function primarily via TLRs, and ligation of these receptors stimulates the secretion of large amounts of cytokines, in particular type I IFN (1, 9, 10). pDCs in humans and rodents express a restricted range of TLRs, particularly TLR7 and 9 (11–13), and this restricted expression explains their selective responsiveness to ssRNA (14–16) and bacterial (13, 17) and viral DNA (18, 19). *In vitro*, activated pDCs secrete proinflammatory cytokines, change their morphology from a round cell to dendritic cell (DC)-like, up-regulate MHC and costimulatory molecules, and become effective APCs. Thus, human pDCs activated by CD40L or influenza virus can induce proliferation and polarization of T cells (20, 21). Murine pDCs activated through TLR9 can also prime CTLs to endogenous, but not exogenous Ag after *i.v.* administration (22).

In addition, pDCs enriched from mice infected with virus have been shown to activate naive CD8<sup>+</sup> T cells (23) and to promote proliferation and polarization of Ag-experienced unpolarized CD4<sup>+</sup> T cells (24). pDCs may also be involved indirectly in the induction of CTLs by helping classical DCs through secretion of cytokines and also through pDC-DC interactions (23, 25).

pDCs may have a role in tolerogenic responses, as they can induce development of anergic T cells or T cells with regulatory functions *in vitro* (26–30). In addition, pDCs have been shown to facilitate allogeneic hemopoietic stem cell engraftment as well as being essential for tolerance to vascularized cardiac allografts (31, 32). It has been shown recently that depletion of pDCs can lead to airway hyperreactivity to normally inert inhaled Ags, and that adoptive transfer of Ag-loaded pDCs before sensitization could prevent the induced asthma (8). In this study, following Ag inhalation, Ag-bearing pDCs could be isolated from both the lung and the draining LN (DLN). This suggested that similarly to classical DC, pDCs may acquire Ag at mucosal sites and transport it to the DLN, and that in the absence of pathogenic stimuli these pDCs can induce tolerogenic responses (8). However, direct evidence for pDC migration in afferent lymph under steady-state conditions is lacking.

In contrast to most classical DCs, pDCs express CD62L, which permits their attachment to high endothelial venules and subsequent entry into lymphoid tissues under steady-state conditions (5, 33, 34). pDCs also express a restricted range of chemokine receptors (e.g., CXCR3, CXCR4, and CCR5) that together may be important in the increased recruitment of pDCs to inflamed LNs (33, 35–37). In addition, pDCs have been shown to accumulate in the skin of herpes virus-induced skin lesions and to infiltrate the skin of psoriatic patients (38, 39). Although activation of bone marrow-derived murine pDCs via TLR9 up-regulates CCR7 expression and induces increased migration toward CCR7 ligands (40), whether pDCs migrate via afferent lymphatics to the DLN after microbial or inflammatory stimuli in peripheral tissues has not yet been determined *in vivo*.

\*Sir William Dunn School of Pathology, Oxford, United Kingdom; <sup>†</sup>Kings College London, Peter Gorer Department of Immunobiology, London, United Kingdom; and <sup>‡</sup>Division of Cell Biology and Immunology, Wellcome Trust Biocentre, University of Dundee, Dundee, United Kingdom

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<sup>1</sup>Address correspondence and reprint requests to Dr. G. Gordon MacPherson, Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE, U.K. E-mail address: gordon.macpherson@path.ox.ac.uk

<sup>2</sup>Abbreviations used in this paper: pDC, plasmacytoid DC; CoeLNx, celiac lymphadenectomy; CpG-ODN, oligonucleotide containing CpG motifs; DC, dendritic cell; DLN, draining lymph node; iL-DC, intestinal lymph DC; L-DC, lymph DC; LN, lymph node; MLNX, mesenteric lymphadenectomy; R-848, Resiquimod; TDL, thoracic duct leukocyte; int, intermediate.

To determine directly whether pDCs do migrate from peripheral mucosal tissues via afferent lymph, we surgically removed the mesenteric or hepatic LN, resulting in mesenteric lymphadenectomy (MLNX) and celiac lymphadenectomy (CoeLNx), respectively. After allowing time for healing of afferent and efferent lymph vessels, we cannulated the thoracic duct of these rats and collected the leukocytes (thoracic duct leukocytes (TDLs)). By phenotypic and functional comparison of TDLs with the recently identified splenic pDCs, we show that intestinal and hepatic lymph are devoid of pDCs under steady-state conditions and after feeding Resiquimod (R-848), a TLR7/8 ligand.

## Materials and Methods

### Animals and surgical procedures

PVG (RT1<sup>c</sup>) rats were bred and maintained under specific pathogen-free conditions in the Sir William Dunn School of Pathology. Rats used were males of 12–24 wk of age. MLNX, CoeLNx, and thoracic duct cannulation were performed, as described previously (41–43). All procedures were conducted in accordance with Home Office guidelines.

### Reagents

Cell cultures were grown in IMDM with 5% FCS, 50  $\mu$ M 2-ME, 100  $\mu$ g/ml penicillin, and 50 U/ml streptomycin (IMDM-5%) (all obtained from Invitrogen Life Technologies). R-848 (InvivoGen) was dissolved in water.  $\beta$ -propiolactone-inactivated influenza virus, previously described (44), was used. Oligonucleotide containing CpG motifs (CpG-ODN) 2336 was obtained from Coley Pharmaceutical.

### Antibodies

mAbs to rat Ags, Ig $\kappa$  (OX12), CD5 (OX19), CD6 (OX52), CD8 $\alpha$  (OX8), CD11b/c (OX42), CD45RA (OX33), CD45RC (OX22), CD90 (OX7), CD103 (OX62), CD172a (OX41), CD200 (OX2), and CD200R (OX102) were purified from cell culture supernatants and used for depletions or conjugated to biotin, FITC, or Cy5. Purified anti-CD32 (D34-485) and anti-CD36 (CB38), both later biotinylated; PE-labeled anti-CD3 (G4.18), anti-CD4 (OX38), anti-CD45R (HIS24), anti-CD62L (OX85), and anti-CD161 (10/78); PerCP-labeled anti-CD8 (OX8) and anti-MHC-II (OX6); and streptavidin-PE and -allophycocyanin were all purchased from BD Pharmingen. Anti-CD11c (8A2), later biotinylated, and PE-labeled anti-TCR $\gamma\delta$  (V65) were purchased from Serotec. Biotinylated anti-Ig $\lambda$  (MRL-61) was purchased from LCG Bioscience. Biotinylated polyclonal sheep anti-murine Siglec-H was generated, as described previously (45).

### Isolation of cells

TDLs were collected on ice in PBS containing 10 mM EDTA and 20 U/ml heparin, and RBC were lysed.

Spleens were diced and treated for 30 min at 37°C in IMDM-5% with 1 mg/ml collagenase type IV (Roche Diagnostics) and 0.25 mg/ml DNase I (Roche). Cells were then passed through a cell strainer and RBC lysed.

TDLs and splenocytes were depleted of T and B cells using anti-CD6, anti-CD8, and anti-CD45RA, followed by goat anti-mouse Dynabeads (DynaL Biotech). In functional experiments, the cell populations were purified further by cell sorting using a MoFlo (DakoCytomation). Purity of the cell populations was then >96%.

### FACS

Surface staining for FACS was performed in PBS with 2% FCS and 10 mM EDTA for 15 min on ice after blocking in 10% rat serum. Cells were then fixed in 2% paraformaldehyde and analyzed using a FACSCalibur (BD Biosciences).

### Polymerase chain reaction

Cells were suspended, and the RNA was extracted in TRIzol (Invitrogen Life Technologies). RNA was treated with the DNasefree kit (Ambion) and RNasin (Promega) and reverse transcribed using the Reverse-IT RT system (ABgene). The amount of cDNA was normalized to cyclophilin B by non-saturating PCR. Sequences of the primers were as follows: BCR, forward ACACGGCTGTGTATTACTACTGT, reverse GTGACCAGGGTACCTGGCCCCAG; cyclophilin B, forward CAAGACCTCTGGCTA GACG, reverse GCTGTCCGCTCTGGTGTCT. Reactions were analyzed by gel electrophoresis.

### Real-time PCR

All quantitative PCR were performed in 20- $\mu$ l vol using 1 U of *Taq* polymerase (HotStar *Taq*; Qiagen) per reaction. Cycling parameters were as follows: 92°C for 30 s, followed by annealing at 60°C for 30 s and extension at 72°C for 15 s for 50 cycles. Real-time PCR data were collected by the ABI 7900HT Sequence Detection System (Applied Biosystems) and analyzed for relative expression of different genes by the SDS 2.2 software (Applied Biosystems).

Real-time PCRs for TNF- $\alpha$ , IFN- $\beta$ , and cyclophilin B were performed using dual-labeled fluorescent probes carrying a 6-FAM reporter dye at the 5' end and a TAMRA quencher at the 3' end. Real-time PCR for IFN- $\alpha$  was performed in the presence of SYBR Green I dye (Molecular Probes). Sequences of the primers were as follows: cyclophilin B, forward CAA GACTCCTGGCTAGACG, reverse GCTGTCCGCTCTGGTGTCT, probe AAAGTTCTGGAAGGCATGGATGTGGTACG; IFN- $\beta$ , forward CCTCTCCATCGACTACAAGC, reverse TGAGGTTGAGCCTCCATT, probe ACAAAAGCACTAGCATTCCGACATGTCAGAA; TNF- $\alpha$ , forward CAGCAGATGGGCTGTACCTT, reverse TGGTATGAAGTGGCAAATCG, probe CTATGTGCTCTCACCCACACCGTC; and IFN- $\alpha$ , forward CAGCA GATCCAGAAGDCTCAARC, reverse CAGGCACAGGGRCTGTGTTT.

### Quantitation of cytokine production

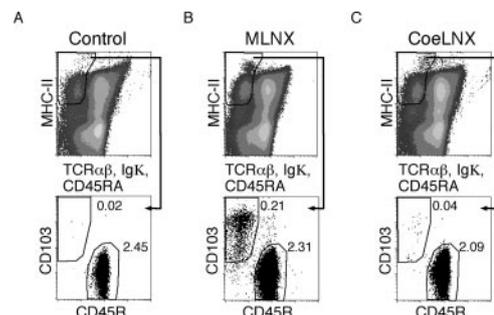
ELISAs for rat IL-6 (BD Pharmingen) and IL-12p40 (BioSource International) were performed, according to manufacturer's protocols.

## Results

### Efferent lymph as well as pseudoafferent intestinal and hepatic lymph contains MHC-II<sup>+</sup>CD45R<sup>+</sup>CD45RA<sup>-</sup>TCR $\alpha\beta$ <sup>-</sup> cells

To determine whether pDCs migrate in efferent lymph to blood under steady-state conditions, we collected TDLs from nonlymphadenectomized rats. No rat pDC-specific mAb is available, but pDCs have been characterized recently in rat spleen as being MHC-II<sup>+</sup>CD45R<sup>+</sup>CD45RA<sup>-</sup>CD3<sup>-</sup>CD103<sup>-</sup> (12). TDLs were therefore stained for MHC-II, CD45RA, TCR $\alpha\beta$ , and Ig $\kappa$ , and analyzed by flow cytometry (Fig. 1A, upper density plot). Cells expressing MHC-II, but not Ig $\kappa$ , CD45RA or TCR $\alpha\beta$ , were gated and analyzed for the expression of CD45R and CD103. Using this gating strategy, we could identify a candidate pDC population in TDLs that constituted 2–2.5% of total TDLs and was MHC-II<sup>+</sup>CD45RA<sup>-</sup>TCR $\alpha\beta$ <sup>-</sup>Ig $\kappa$ <sup>-</sup>, with almost all cells being CD45R<sup>+</sup>CD103<sup>-</sup> (Fig. 1A). The same population of cells, present with similar frequency, was detected when CD45RA was omitted from the staining, showing that no potential pDCs were being gated out when using this mAb (data not shown).

Classical DCs migrate from peripheral tissues in afferent lymph, but almost all are filtered out in DLN. To determine whether pDCs behave similarly, we removed MLNs or the liver-draining celiac



**FIGURE 1.** Analysis of MHC-II<sup>+</sup>TCR $\alpha\beta$ <sup>-</sup>CD45RA<sup>-</sup>Ig $\kappa$ <sup>-</sup> TDLs in efferent, pseudoafferent hepatic, and intestinal lymph. TDLs from control (A), CoeLNx (B), or MLNX (C) rats were stained and analyzed by flow cytometry. MHC-II<sup>+</sup>TCR $\alpha\beta$ <sup>-</sup>CD45RA<sup>-</sup>Ig $\kappa$ <sup>-</sup> cells were then gated out, as indicated by the arrows, and further analyzed for the expression of CD45R and CD103. The numbers in the plots represent the percentage of cells (within the indicated gates) of total TDLs.

LN from young rats, creating MLNX and CoeLNX rats, respectively. By 6 wk, the afferent and efferent lymph vessels have anastomosed, which permits collection of pseudoafferent intestinal or liver lymph cells from the thoracic duct (41–43). As found in nonlymphadenectomized rats, we could identify a population of MHC-II<sup>+</sup>CD45RA<sup>-</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs, in both MLNX and CoeLNX lymph, that was CD45R<sup>+</sup>, but expressed no CD103 and constituted 2–2.5% of all TDLs (Fig. 1, B and C). As expected, MLNX and CoeLNX lymph also contained a population of MHC-II<sup>+</sup>CD45RA<sup>-</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> cells, but that in contrast was CD45R<sup>-</sup>CD103<sup>+</sup>, representing classical migrating lymph DC (L-DC) (Fig. 1, B and C) (43, 46). This population was virtually absent in nonlymphadenectomized rats, as in these rats almost all L-DCs are trapped in the DLN (Fig. 1A).

These experiments show that thoracic duct lymph contains cells that are MHC-II<sup>+</sup>CD45R<sup>+</sup>CD45RA<sup>-</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> and that these cells are present at similar frequencies in pseudoafferent and efferent lymph.

#### Intestinal MHC-II<sup>+</sup>Igκ<sup>-</sup>CD45RA<sup>-</sup>TCRαβ<sup>-</sup>CD45R<sup>+</sup> TDLs differ phenotypically from splenic pDCs

To determine whether the MHC-II<sup>+</sup>CD45R<sup>+</sup>CD45RA<sup>-</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs were pDCs, we performed a more detailed phenotypic analysis of lymph cells from MLNX rats. These cells were compared with authentic splenic pDCs isolated by collagenase treatment (12). Intestinal TDLs and splenocytes were depleted of CD6<sup>+</sup>, CD8<sup>+</sup>, and CD45RA<sup>+</sup> cells using magnetic beads, and MHC-II-expressing cells were analyzed by flow cytometry (Fig. 2A, density plots). In addition, mAbs to TCRαβ and Igκ were included in the staining, and any remaining stained cells were gated out. TDL and splenocyte MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> populations were further separated by staining with CD45R (Fig. 2A). In both TDL and spleen, the MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup>CD45R<sup>-</sup> cells also expressed CD103, CD11b/c, and CD11c, confirming their identity as classical intestinal lymph-DCs (iL-DCs) (43, 46) and lymphoid tissue DCs, respectively (47) (Fig. 2A, upper row of histograms). In contrast, the CD45R<sup>+</sup> cells from both tissues did not express CD11b and c, and expressed very low, if any, CD103 (Fig. 2A, lower row of histograms). As all MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> cells are CD45R<sup>+</sup>, given the low expression of some surface molecules and

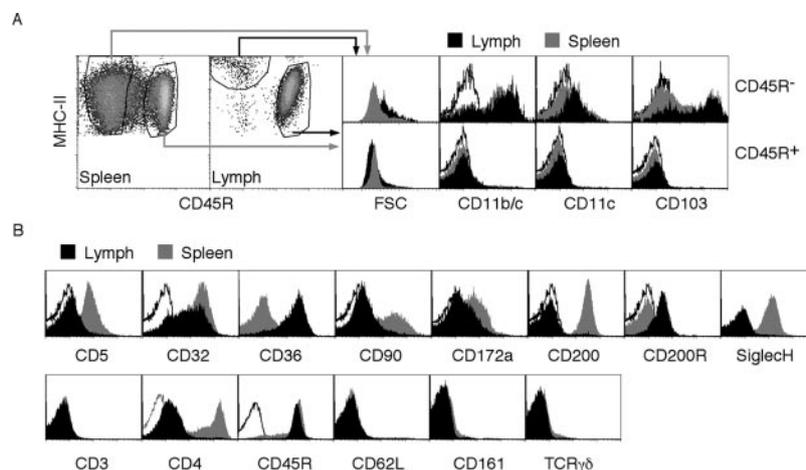
lack of conjugated Abs, in some stainings we used anti-CD45RC in place of CD45R (Fig. 2B, lower row of histograms).

Splenic pDCs (MHC-II<sup>+</sup>CD45R(C)<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup>) expressed high levels of CD4, were positive for CD5, CD32, CD90, CD172a, and CD200<sup>+</sup>, but were negative for CD36 and CD200R (Fig. 2B). In contrast, the candidate lymph pDCs (MHC-II<sup>+</sup>CD45R(C)<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup>) were CD4<sup>low</sup>, CD32<sup>int</sup>, CD90<sup>low</sup>, and CD172a<sup>low</sup>, expressed CD36 and CD200R, but were negative for CD5 and CD200 (Fig. 2B). Neither splenic nor TDL populations expressed TCRγδ, CD3, or CD161. The surface phenotype of the MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> splenocytes is identical with the recently described splenic pDCs (12), except that we did not detect expression of CD161 (Fig. 2B). A small population of CD161<sup>+</sup> NK cells was detected in lymph, but these were phenotypically distinct from the MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> cells (data not shown).

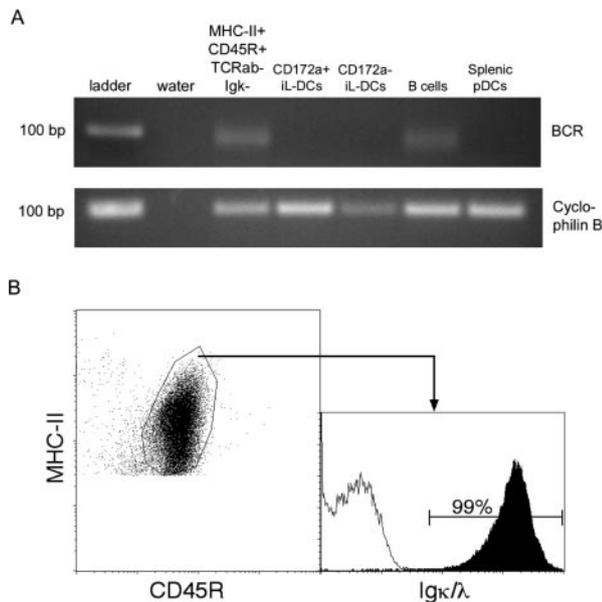
Murine pDCs have been shown recently to express high levels of Siglec-H selectively (45). A biotinylated polyclonal Ab directed against murine Siglec-H (45, 48) was therefore used to stain the lymph and splenic MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> populations. Although no staining of the TDL population could be detected, splenic pDCs were Siglec-H<sup>+</sup> (Fig. 2B). No staining was observed when the biotinylated prebleed serum was used (data not shown).

Analysis by PCR showed rearrangement of VDJ genes in the TDL population (Fig. 3A). In contrast, no VDJ rearrangement was detected in sorted splenic pDCs. iL-DCs (both CD172a<sup>+</sup> and CD172a<sup>-</sup>) were also negative for VDJ rearrangement, whereas such rearrangement was easily detectable in splenic B cells (Fig. 3A). Apart from CD45RA, there is no pan-B cell marker available in rat, as no Abs to CD19 have been generated. In addition, many of the polyclonal sera directed against rat Ig have weak reactivity to subclasses other than IgG (our unpublished observation). The MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>CD45RA<sup>-</sup> TDLs were therefore analyzed for surface Ig using a combination of mAbs against κ and λ L chain. When these reagents were combined, 99% of the gated TDLs were positive (Fig. 3B).

These data show that rat intestinal TDLs contain a population of MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>CD45RA<sup>-</sup> cells, but that these differ from splenic pDCs in the expression of a number of surface markers,



**FIGURE 2.** Phenotypic analysis of MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs and splenocytes. *A*, Splenocytes or TDLs from MLNX rats were depleted of CD6<sup>+</sup>, CD8<sup>+</sup>, and CD45RA<sup>+</sup> cells. MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> cells were then further analyzed for CD45R expression by flow cytometry (density plots). The upper row of histograms shows expression of surface markers by CD45R<sup>-</sup>, and the lower shows CD45R<sup>+</sup> splenocytes (gray-filled histograms) or TDLs (black-filled histograms) gated from the density plots, as indicated by the arrows. The open histograms represent staining with an isotype control. *B*, Histograms show expression of surface markers by MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> splenocytes or TDLs gated in addition on CD45RC<sup>+</sup> (lower row) or CD45R<sup>+</sup> (upper row of histograms) as in the lower histogram of *A*.



**FIGURE 3.** Expression of Ig by cell populations sorted from intestinal lymph or spleen. *A*, After enrichment by bead depletion of CD6<sup>-</sup>, CD8<sup>-</sup>, and CD45RA-expressing cells, the cell populations were sorted by MoFlo into MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRab<sup>-</sup>Igκ<sup>-</sup> intestinal TDLs, CD172a<sup>+</sup> or CD172a<sup>-</sup>iL-DCs, splenic pDCs, or splenic MHC-II<sup>+</sup>CD45R<sup>+</sup>CD4<sup>+</sup>TCRab<sup>-</sup>Igκ<sup>-</sup>pDCs followed by semiquantitative PCR of rearranged Ig genes. *B*, TDLs from MLNX rats were depleted of CD6<sup>+</sup>, CD8<sup>+</sup>, and CD45RA<sup>+</sup> cells and stained for flow cytometry. MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRab<sup>-</sup>Igκ<sup>-</sup> TDLs were then gated out and analyzed in the dot plot. CD45R-expressing TDLs were finally gated out, as indicated by the arrows, and analyzed in the histogram for the expression of Igκ/Igλ. The open histogram represents staining with the isotype controls.

including Siglec-H. In contrast to splenic pDCs, the TDL population also expresses Ig, which shows that the vast majority of these cells are B cells, despite their lack of CD45RA expression.

#### *Intestinal MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs do not respond to viral stimuli in vitro*

Functional characteristics of pDCs include cytokine secretion, changes in cell morphology, and increased survival in response to microbial stimuli. In particular, rat splenic pDCs express high levels of TLR7 and 9, making them responsive to ssRNA, bacterial and viral DNA (12). To determine whether TDLs from intestinal lymph contained cells that were functionally similar to pDCs, MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs and splenic pDCs were sorted and stimulated *in vitro*. Incubation of the sorted TDLs for 24 h with R-848, a synthetic TLR7/8 ligand, or CpG-ODN induced a slight increase in survival (1.4- to 1.9-fold), but no change in survival was seen after incubation with β-propiolactone-inactivated influenza virus (Fig. 4A). In marked contrast, splenic pDCs showed considerably increased survival after incubation with R-848 or CpG-ODN, and strikingly, splenic pDCs displayed a 6- to 7-fold increase in survival after incubation with inactivated influenza virus. Such stimulation also caused splenic pDCs to become larger and more DC-like by 24–36 h, whereas no such changes were observed in the sorted TDL population.

A hallmark of pDCs is the rapid production of inflammatory cytokines, in particular type I IFNs, in response to viral stimuli. We therefore cultured sorted MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs and splenic pDCs for 5 h with the stimuli described above. Splenic pDCs showed a 100- to 150-fold increase in IFN-α and a 300- to 500-fold increase in IFN-β mRNA levels in response to inactivated influenza virus or CpG-ODN (Fig. 4, B and C). In

addition, these cells displayed a 3000- and 9000-fold increase in TNF-α mRNA levels after incubation with influenza virus or CpG-ODN (Fig. 4D). R-848 also induced increased expression of all three cytokines, albeit to a lower level (Fig. 4, B–D). In complete contrast, the sorted intestinal TDLs showed no increase in IFN-α, IFN-β, or TNF-α mRNA levels in response to any of the stimuli (Fig. 4, B–D). Moreover, when secretion of IL-12p40 and IL-6 was measured after 24 h in culture, no cytokines were detected in TDL cultures, whereas high levels of both cytokines were present in splenic pDCs cultures, in particular after R-848 and CpG-ODN stimulation (Fig. 4, E and F). There is currently no commercial ELISA for rat IFN-α, and to our knowledge no IFN-α-blocking reagent. Preliminary experiments have, however, shown that supernatants collected from splenic pDCs, but not from the sorted TDLs cultured with R-848, CpG-ODN, and inactivated influenza virus significantly inhibit infection of a rat kidney cell line by Semliki Forest virus, a property of type I IFNs (data not shown). This shows that whereas splenic pDCs respond rapidly to viral stimuli as assessed by increased survival and cytokine secretion, no such response occurs with the MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs.

These data show that under steady-state conditions, neither efferent nor pseudoafferent intestinal lymph contains cells with pDC characteristics.

#### *Per-oral TLR7/8 stimulation does not induce pDC release into afferent intestinal lymph*

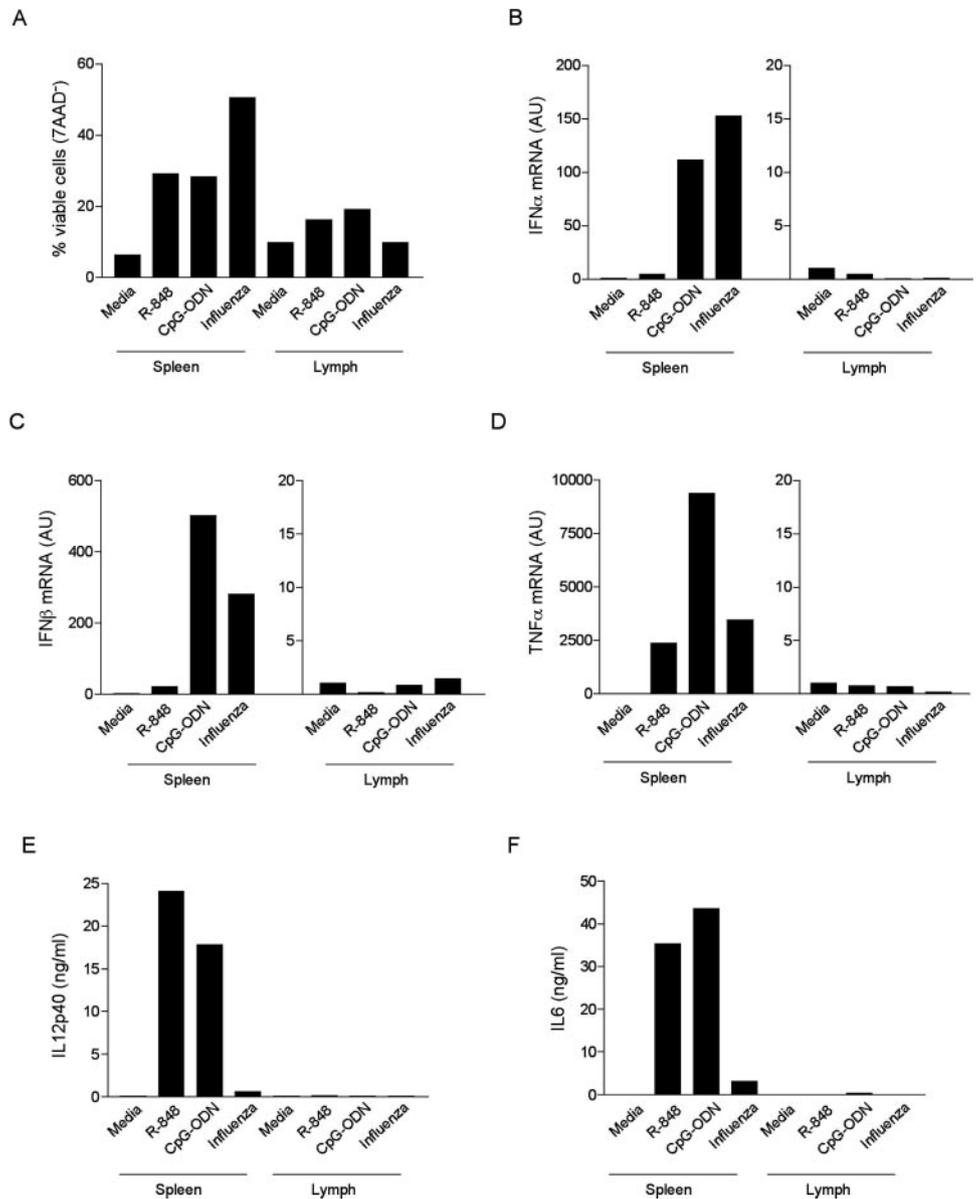
pDCs are recruited to inflamed LNs and redistribute within LNs in response to systemic viral stimuli (9). In addition, feeding mice R-848 results in activation and secretion of cytokines by pDCs in gut-associated lymphoid tissues (49). pDCs have been characterized in murine liver, and in preliminary experiments we have identified pDC-like cells in rat livers (data not shown) (6). As pDCs have been identified in peripheral mucosal tissues such as the lung (8), it was important to determine whether intestinal pDCs were released into lymph after activation *in vivo*. We have shown that oral R-848 induces a rapid, but transient 30- to 50-fold increase in the output of MHC-II<sup>+</sup>Igκ<sup>-</sup>CD45R<sup>-</sup> iL-DCs into lymph (49) (Fig. 5A). We thus fed R-848 to MLNX rats and collected lymph over a period of 18 h. MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs almost completely disappeared from lymph during this period, as also happens with B and T lymphocytes (Fig. 5A). Importantly, when MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs were sorted from R-848-fed rats and cultured, they did not secrete IL-12p40 or IL-6, whereas these cytokines were actively secreted by iL-DCs sorted from the same rats (Fig. 5B).

These data show that feeding a TLR7/8 ligand does not stimulate pDCs to enter afferent intestinal lymph.

## Discussion

pDCs recognize pathogen-associated molecules and represent an important component of innate defense, most likely via cytokine secretion, but possibly also as APCs. It has been suggested recently that these cells may also have important functions in sustaining tolerance to innocuous Ags at mucosal sites (8). pDCs have been identified in blood, mucosal, and lymphoid tissues of rodents under steady-state conditions. The central question of how, or whether pDCs migrate between these tissues has not, however, been assessed directly. To determine whether pDCs migrate from LNs via efferent lymph to blood, we cannulated the thoracic ducts of nonlymphadenectomized rats and characterized TDLs not expressing pan-T, -B, and -NK cell markers. To determine whether pDCs migrate from peripheral sites to the DLN, we collected lymph cells from pseudoafferent intestinal and hepatic lymph.

**FIGURE 4.** Cytokine production in vitro by MHC-II<sup>+</sup>TCR $\alpha\beta$ <sup>-</sup>Ig $\kappa$ <sup>-</sup> TDLs and splenocytes. TDLs from MLNX rats or splenic pDCs were depleted of CD6-, CD8-, and CD45RA-expressing cells. A total of  $5 \times 10^4$  MHC-II<sup>+</sup>TCR $\alpha\beta$ <sup>-</sup>Ig $\kappa$ <sup>-</sup> TDLs and splenocytes was then sorted by MoFlo and cultured in medium or in the presence of R-848 (1  $\mu$ g/ml), CpG-ODN (3  $\mu$ g/ml), or inactivated influenza virus ( $4 \times 10^3$  hemagglutinating units) for 5 h (B–D) or 24 h (A, E, and F). The cells cultured for 5 h were resuspended in TRIzol, the RNA was extracted, and after reverse transcription the levels of IFN- $\alpha$  (B), IFN- $\beta$  (C), and TNF- $\alpha$  (D) mRNA relative to the medium controls were determined by quantitative PCR. Cells cultured for 24 h were stained for 7-aminoactinomycin D (7AAD) (A) to determine viability, and the supernatants were screened for IL-12p40 (E) and IL-6 (F) by ELISA. These results are representative of two independent experiments with TDLs and three with splenic pDCs. AU, Arbitrary units.

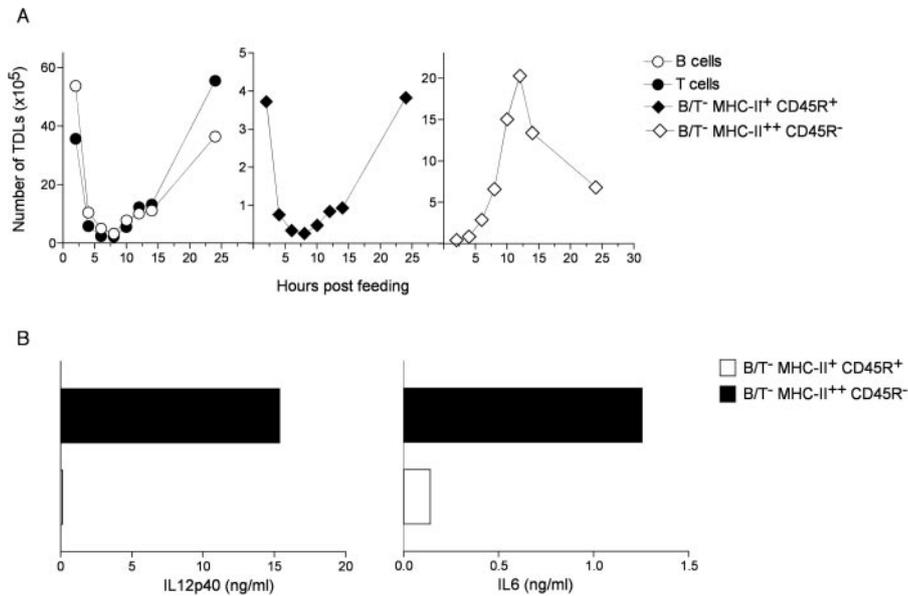


In efferent lymph and in hepatic and intestinal pseudoafferent lymph, we detected TDLs that express CD45R and MHC-II, but not CD45RA, TCR $\alpha\beta$ , or CD161. In addition, these cells were negative for CD103 or CD11b/c, markers expressed by rat DCs and monocytes/macrophages, respectively. Although there is no specific marker for rat pDCs, the phenotype of these cells is somewhat similar to that recently described for rat splenic pDCs (12). However, a direct phenotypic comparison of this TDL population and splenic pDC showed that these cells differ in the expression of a number of other surface markers. In particular, splenic pDCs express CD4, CD5, CD90, and CD200, whereas the TDL population expressed no or low levels of these surface Ags. In addition, >99% of the TDL population is surface Ig<sup>+</sup>, whereas the splenic pDCs do not contain rearranged VDJ genes. Finally, the splenic pDCs are Siglec-H<sup>+</sup>, a surface receptor recently described as being selectively expressed by murine pDCs (45, 48), whereas no expression could be detected on the TDLs. This phenotypic characterization strongly suggests that intestinal lymph is devoid of cells with a pDC phenotype.

To investigate whether cells with functional characteristics of pDCs could be detected in intestinal lymph, TDLs expressing

CD45R and MHC-II, but not TCR $\alpha\beta$  or Ig $\kappa$ , were purified. The hallmark of pDCs, in humans as well as rodents, is the rapid production of cytokines in response to viral stimuli. When, however, the sorted TDLs were stimulated in vitro with inactivated influenza virus or CpG-ODN and R-848, TLR9 and TLR7/8 ligands, no increased transcription of either type I IFNs or TNF- $\alpha$  could be observed, nor was IL-6 or IL-12p40 secreted. In marked contrast, splenic pDC stimulated identically showed increased transcription of type I IFNs and TNF- $\alpha$  as well as secretion of IL-6 and IL-12p40. These results show that efferent and intestinal and hepatic afferent lymph under steady-state conditions are devoid of bona fide pDCs. As we could not detect any pDCs in efferent or pseudoafferent lymph, these experiments demonstrate that pDCs that have entered LNs from blood do not subsequently leave via lymph to go to other lymphoid organs.

It has been suggested previously that pDCs, just as conventional DCs, migrate from the lung to the DLN under steady-state conditions, as inhaled Ags were found to be associated with pDCs both in the lungs and DLN (8). In addition, in the same study, adoptively transferred Ag-loaded pDCs prevented the development of Ag-induced asthma. Our results show, however, that pDCs are not



**FIGURE 5.** Migratory and secretory response of MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs to a fed TLR7/8 ligand. Thoracic ducts of MLNX rats were cannulated, and animals were fed PBS or R-848 (50 μg/rat) by gavage. **A**, TDLs were collected, and the output over 2-h periods was determined. These TDLs were then stained for TCRαβ, Igκ, MHC-II, and CD45R, and the frequency of TCRαβ<sup>+</sup> T cells (●), MHC-II<sup>+</sup>Igκ<sup>+</sup> B cells (○), MHC-II<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> TDLs (◆), and MHC-II<sup>++</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> iL-DCs (◇) was determined and used to calculate the output of each subset of cells. **B**, TDLs collected for 18 h after feeding R-848 were sorted by MoFlo into MHC-II<sup>+</sup>CD45R<sup>+</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> or MHC-II<sup>++</sup>CD45R<sup>-</sup>TCRαβ<sup>-</sup>Igκ<sup>-</sup> (iL-DCs) and cultured in medium for 24 h. The supernatants were screened for IL-12p40 and IL-6 by ELISA. These results are representative of three independent experiments.

present in afferent lymph draining another mucosal tissue, namely the intestine. Whether pDCs are present in the intestine is still controversial, and it is possible that the lack of pDCs in intestinal lymph reflects their absence from the intestine under steady-state conditions. The liver, however, does contain pDCs in rodents (6), but no pDCs could be identified in afferent liver lymph. These experiments thus strongly suggest that, unlike classical DCs, pDCs present in mucosal tissues and liver do not induce tolerogenic or immunogenic Ag-specific T cell responses by acquiring Ag in the periphery and transporting it via afferent lymph to the DLN. How pDCs in LN do acquire peripheral Ags thus remains unknown. Potential mechanisms include delivery by classical DCs or other mechanisms such as exosomes or release of DC fragments (50, 51).

pDCs have been shown to be recruited from blood via high endothelial venules to inflamed lymphoid tissues (37). Importantly, they are also enriched in peripheral tissues during immune reactions, as, for example, they infiltrate the skin of psoriatic patients (39). pDCs express TLR7 and systemic as well as oral administration of the TLR7/8 ligand, R-848, induces pDC activation, cytokine secretion and migration within lymphoid tissues (9, 49). However, we could not detect pDCs either in intestinal lymph after feeding R-848 or in liver lymph after systemic administration (U. Yrlid and G. MacPherson, unpublished observation). It has been reported that murine splenic pDCs activated by a viral stimulus in vitro or in vivo, following adoptive transfer acquire a phenotype similar to that of classical DCs, expressing high levels of MHC-II and CD11c (34). Importantly, in contrast to classical DCs, these activated pDC retain expression of CD45RA. At no time after feeding R-848 could we, however, detect iL-DCs that expressed enhanced levels of CD45R, neither did sorted rat splenic pDC stimulated in vitro with R-848 modulate their expression of CD11b/c, CD103, or CD45R (U. Yrlid and G. MacPherson, unpublished observation).

In this study using our unique rat model, we have shown that efferent as well as intestinal and hepatic afferent lymph are devoid

of cells with the phenotype and function of pDCs, both under steady-state conditions and after TLR7/8 administration. These experiments show that pDCs present in the intestine or liver do not share the migration pattern of classical L-DCs, and the potential function of pDCs as APCs can therefore not result from uptake and transport of Ags to the DLN. These results have important implications for future strategies using adoptive transfer of pDCs for induction of Ag-specific immune responses.

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## Disclosures

The authors have no financial conflict of interest.

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