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Functional Specialization of Islet Dendritic Cell Subsets

Na Yin,*1 Jiangnan Xu,*4,‡ Florent Ginhoux,§,2 Gwendalyn J. Randolph,§ Miriam Merad,§ Yaozhong Ding,*4,‡ and Jonathan S. Bromberg*4,‡

Dendritic cells (DC) play important roles in both tolerance and immunity to β cells in type 1 diabetes. How and why DC can have diverse and opposing functions in islets remains elusive. To answer these questions, islet DC subsets and their specialized functions were characterized. Under both homeostatic and inflammatory conditions, there were two main tissue-resident DC subsets in islets, defined as CD11b+CD103+CX3CR1+ (CD103+ DC), the majority of which were derived from fms-like tyrosine kinase 3-dependent pre-DC, and CD11b+CD103−CX3CR1+ (CD11b+ DC), the majority of which were derived from monocytes. CD103+ DC were the major migratory DC and cross-presented islet-derived Ag in the pancreatic draining lymph node, although this DC subset displayed limited phagocytic activity. CD11b+ DC were numerically the predominant subset (60–80%) but poorly migrated to the draining lymph node. Although CD11b+ DC had greater phagocytic activity, they poorly presented Ag to T cells. CD11b+ DC increased in numbers and percentage during T cell-mediated insulitis, suggesting that this subset might be involved in the pathogenesis of diabetes. These data elucidate the phenotype and function of homeostatic and inflammatory islet DC, suggesting differential roles in islet immunity. The Journal of Immunology, 2012, 188: 4921–4930.

Islets contain resident dendritic cells (DC) that play important roles in type 1 diabetes and allogeneic islet transplant rejection. Both protective and pathogenic roles for DC in type 1 diabetes have been reported (1–5). DC are a heterogeneous population that consists of different subsets with distinct developmental, phenotypic, and functional properties. Several groups have shown that NOD mice have an imbalance in the numbers of specific splenic DC subsets, with a relative decrease in the number of CD8+ DC and an increase in myeloid DC (4). Characterization of islet DC subsets in NOD has not been reported.

Nonlymphoid tissue DC capture Ag and migrate from the periphery to the draining lymph node (LN), where they initiate immune responses, and deliver cues that influence T cell effector function. In the periphery, tissue-resident DC phagocytose apoptotic cells generated during normal tissue turnover, migrate to draining LNs, and present Ags derived from the apoptotic cells (5, 6). This presentation of self-Ags under steady-state conditions is thought to lead to deletion or anergy of self-reactive T cells, thereby maintaining T cell tolerance. Distinct DC subsets have been identified in nonlymphoid tissues and can be categorized, based on the surface markers CD103, CD207, CD11b, and CD103 (CD103+ DC), the majority of which were derived from fms-like tyrosine kinase 3 (Flt3) ligand and its receptor Flt3, whereas CD103− DC are a heterogeneous population dependent on both Flt3 and M-CSF receptor (7–9). The two DC subsets have different functions in the intestine and lung (8, 9, 11, 12). Lamina propria CX3CR1+CD103+ DC sample intestinal Ags by projecting dendrites through the epithelial cell layer and into the lumen may serve as a first line of defense by phagocytosing and killing bacteria (13–16). CD103+ DC from gut-associated mesenteric LNs produce endogenous TGF-β and retinoic acid (RA) and are capable of differentiating naive T cells into Foxp3+ regulatory T cells (Tregs) independent of exogenous TGF-β (17–21). Lung-migrating CD103+ DC are the major contributors in cross-presentation for CD8+ T cells under tolerogenic conditions (22) and for activation following poxvirus infection (23). Dermis-derived CD103+ DC but not CD103− DC constitutively produce RA and induce adaptive Tregs (24). Thus, the heterogeneity of DC highlights their functional versatility in shaping local tissue immunity and their collaboration in orchestrating immune responses. These subsets are also found in islets (7), but their functional roles have not been tested.

Studies have shown that APCs in islets present β cell-derived peptides to their MHC class II (MHCII) molecules (25). T cell-mediated inflammation induces islet DC maturation, which leads to further processing of captured Ags (26). However, in those reports, separate DC subsets were not characterized, and whether they had distinct functions was not explored. In this study, we characterized two major DC subsets in islets as well as their origins and specialized functions during both the steady-state and inflammation. CD103+ DC were the major migratory DC subset and responsible for cross-presenting Ags to CD8+ T cells. CD11b+ DC were the major phagocytic cells whose number was significantly increased during the islet inflammation. Our studies uncovered islet DC heterogeneity, which contribute to an understanding of the mechanisms that balance islet inflammation and tolerance.
Materials and Methods

**Mice**

BALB/c, C57BL/6, MIP-GFP, RIPmOVA, CD11c-cre-GFP, CCR7−/−, Plt, C57BL/6/OT1, and C57BL/6/OT2 mice were from The Jackson Laboratory (Bar Harbor, ME). C3XR1−/− mice on the C57BL/6 and BALB/c backgrounds were from Dr. D. Litman (Skirball Institute, New York, NY). C3XR1−/− and C57BL/6 mice were crossed with C57BL/6 mice to produce C57BL/6×C3XR1−/− F1 hybrids. C57BL/6 mice (27) were provided by Dr. M. Merad (Mount Sinai School of Medicine, New York, NY). LysM-Cre × Rosa26-stopfloxEGFP mice were provided by Dr. G. Randolph (Mount Sinai School of Medicine). All mice were housed in a pathogen-free animal facility. All experimental protocols were approved by the Institutional Animal Care and Utilization Committee of University of Maryland Medical Center.

**Cell preparations**

Mice were euthanized, the common bile duct was exposed and injected with 3 ml cold Hanks’ buffer containing 1.5 mg/ml collagenase-P (Roche Diagnostics, Indianapolis, IN), the pancreas was excised, and digestion was allowed to continue at 37 °C for 15 min. The digested pancreas was disrupted by triturating, and the suspension was washed twice with RPMI 1640 medium containing 10% FBS. Pancreatic islet separation was performed by centrifugation on a discontinuous Ficoll (Sigma-Aldrich, St. Louis, MO) gradient of 11, 21, 23, and 25%. Islets were picked from the interface between the first and second layers. Islets or LNs were incubated for 30 min in 10% FBS in HBSS containing 0.2 mg/ml collagenase type IV (working activity of 770 U/mg; Sigma-Aldrich) then homogenized and passed through a 19g syringe to obtain a single-cell suspension.

**Flow cytometry**

Pancreatic islet cells were resuspended in PBS containing 1% BSA, 2 μg/ml Fc-blocking buffer (eBioscience, San Diego, CA) and 2 mM EDTA and stained with Abs at 4 °C. Flow cytometric analyses were performed on an LSRII flow cytometer (eBioscience) with FlowJo software (Tree Star, Ashland, OR). Dead cells were excluded by DAPI (Invitrogen, Carlsbad, CA) staining. Fluorochrome- or biotin-conjugated mAbs specific to mouse B220 (RA3-6B2), CD8α (53-6.7), MHCII (M5/114.15.2), IAα (IAa, 10-3.6), CD103 (2E7), CD11b (M1/70), CD11c (N418), CD45 (30F11), Gr-1 and subset of DC or dispersed islet or LN cells in 200 μl complete medium with or without 2 ng/ml TGf-β (eBioscience) in 96-well round-bottomed plates. After 5 d, cells were analyzed by flow cytometry. Intracellular staining for Foxp3 expression was performed following the manufacturer’s protocol (eBioscience). Intracellular cytokine (IL-4, IL-17, and IFN-γ) staining was performed after restimulation with PMA (100 ng/ml, Sigma-Aldrich) and ionomycin (500 ng/ml; eBioscience) in the presence of monesin (1/1000 dilution; eBioscience) for 4 h.

**Cell transfers**

Naïve CD4 T cells were isolated with CD4+CD62L− T cell Isolation Kit II (Miltenyi Biotec, Auburn, CA) from OT2 LN and spleen cells and expanded with anti-CD3 (eBioscience)-coated plate and 1 μg/ml anti-CD28 (eBioscience) for 3 d. A total of 2 × 106 OT2 CD4 T cells were transferred to RIPvOVA or littermate C57BL/6 recipients via i.p. injection. CD8+ T cells were enriched from OT1 LN and spleen cells using the CD8α microbeads (Miltenyi Biotec) and expanded with anti-CD3-coated plate and 1 μg/ml anti-CD28 for 3 d. A total of 3.5 × 106 CD8+ T cells were transferred into RIPvOVA or littermate C57BL/6 recipients via i.v. injection.

**Quantitative real-time PCR**

The procedures for RNA isolation, cDNA synthesis, and quantification by quantitative real-time PCR were described previously (29). Quantitative PCR used Oligo(dT) primers on the LightCycler 2.0 (Roche). Relative expression was calculated as 2^-cycle threshold [Ct] control – Ct gene using cyclophilin A as an endogenous control. Primer sequences for CCR7 were 5’-CCACGCCTGATGCTCACTGGA-3’ (forward) and 5’-CCATCTGGG-CXACTTGGA-3’ (reverse).

**OVA uptake assay**

A total of 200 μg Alexa 488-labeled OVA (Invitrogen) were i.v. injected into C57BL/6 mice, and islet single-cell suspensions were assayed by flow cytometry 1.5 and 16 h later. Iset single-cell suspensions were incubated with 2 μg Alexa 488-labeled OVA in 2 ml 10% FBS/RPMI 1640 medium at 37 °C with or without 0.5% sodium azide (NaN3), or on ice for 1 h, and cells were washed three times and assayed at 4 °C by surface marker staining.

**Statistical analysis**

The differences were assessed using the unpaired Student t test and expressed as the mean ± SEM. A p value <0.05 was taken to be statistically significant.

**Results**

**Phenotypes and origins of islet DC during steady state**

Whole-mount immunofluorescent staining of islets showed that a CD31+ blood vessel network was contained within normal naive islets. Most CD11c+CD4+ DC were in close proximity to the surface of intraslit blood vessels (Fig. 1A), suggesting that DC were actively probing the vessel and the surrounding area (25, 30). Next, we analyzed the islet DC subsets. As shown in Fig. 1B, there were two major tissue resident DC (CD11c+MHCII+ subsets) in pancreatic islets during the steady state (7), which we refer to as CD11b+ DC (CD11b+CD103+CX3CR1+F4/80+) and CD103+ DC (CD11b−CD103+CX3CR1−F4/80−). Neither subset expressed CD80 or CD40. Both expressed low levels of CD86 and high levels of MHCII, showing their semimature phenotypes (Fig. 1B). In prediabetic NOD, there also were two major islet DC subsets, CD11b+ DC and CD103+ DC (Fig. 1C). The number of both CD11b+ DC and CD103+ DC was significantly increased in NOD mice, compared with diabetes-resistant NOR mice (Fig. 1C, 1E). Although the percentage and costimulatory molecule expression of these two subsets were similar (Fig. 1D), the CD103 expression level was lower in NOD mice than in NOR mice (Fig. 1C). Next, we determined the origin of these DC subsets. The Fli3 receptor has been reported as a key molecule for the development...
of CD103⁺ DC in most nonlymphoid organs (7, 11). As shown in Fig. 2A and 2B, both the number and percentage of islet CD103⁺ DC were dramatically decreased in Flt3⁻²⁻ mice, whereas the number of CD11b⁺ DC were not affected (7), demonstrating that the development of islet CD103⁺ DC but not CD11b⁺ DC was dependent on Flt3 and derived exclusively from pre-DC.

The possibility that islet steady-state DC were derived from monocytes was tested by using LysM-Cre × Rosa26-floxstop-floxEGFP mice. In these reporter mice, Cre activity removes a stop cassette upstream of the floxed reporter and results in irreversible GFP expression in LysM⁺ cells, including monocytes and their progeny. Although all monocytes express LysM, not all monocytes are GFP⁺ in these mice (31). Therefore, it has been reasoned that DC expressing GFP at levels and percentages comparable to blood monocytes are thus derived primarily from monocytes (31, 32). We found that CD11b⁺ DC had similar GFP expression levels in comparison with blood monocytes, whereas CD103⁺ DC expressed much lower levels of GFP (Fig. 2C, 2D), indicating that the majority of CD11b⁺ DC but not CD103⁺ DC were derived from monocytes under steady-state conditions.

CD103⁺ DC are the major migratory DC

Tissue-draining LN contain tissue migratory and resident DC (33). Migration of DC from tissue to the draining LN is dependent on CCR7 (34, 35). Islet CD103⁺ DC but not CD11b⁺ DC expressed CCR7 (Fig. 3A), suggesting only the CD103⁺ subset had the potential to migrate to draining LN, and the distribution of islet DC subsets was not altered by CCR7 or its ligand deficiency (Fig. 3B).
In the pancreatic draining LN, there were three DC populations, which were CD11b+CD103-, CD11b+CD103+, and a third population of CD11b+CD103+ DC. Neither CD11b-CD103- nor CD11b+CD103+ pancreatic LN DC expressed CX3CR1, which was expressed on the islet CD11b+ DC subset (Figs. 1B, 3C), suggesting that both LN CD11b+CD103+ DC and the third population CD11b+CD103+ DC were derived from islet CD103+ DC (11). Significantly, both CD103+ DC subsets were significantly reduced in the pancreatic LN of CCR7−/− mice in the steady state, whereas the pancreatic LN CD11b+CD103+ DC remained unaltered in these mice (Fig. 3C, 3D). Taken together, these data strongly suggested that islet CD103+ DC activity migrated from islets to LN and were the major migratory DC subset.

**Steady-state Ag presentation: migratory CD103+ DC present β cell-derived Ag, whereas CD11b+ DC have poor Ag presentation ability**

It has been demonstrated that OT1 cells, recognizing an MHC class I (MHCI)-OVA peptide complex, can accumulate and proliferate in the pancreatic draining LN after adoptive transfer into RIPmOVA mice (36, 37), in which OVA is expressed on the membrane of β cells (37), and DC are responsible for the cross-presentation of OVA (36). To recapitulate this cross-presentation, ex vivo, naïve OT1 cells were cocultured with islet cells or pancreatic LN cells. Few OT1 cells proliferated when cocultured with islet cells either from C57BL/6 mice or from RIPmOVA mice. However, OT1 cells proliferated significantly when cocultured with pancreatic LN cells from RIPmOVA mice, which was not observed when cocultured with pancreatic LN cells from C57BL/6 mice (Fig. 4A), demonstrating that resident islet DC were not able to stimulate OT1 cells in islets, but some DC sampled islet-derived Ag, migrated to draining LN, cross-presented the Ag, and stimulated CD8+ T cells. Because CD11b+ DC have limited capability for migrating to LN, we reasoned that CD103+ DC might deliver the islet-derived Ag to LN. To confirm which DC subsets cross-present OVA peptide, we examined the H-2Kb-OVA257–264 complex on the DC subsets of RIPmOVA mice by using a specific Ab that recognizes the peptide–MHCI complex. Low levels of peptide–MHCI complexes were detected on both of the two CD103+ DC subsets from pancreatic LN, whereas the complexes were not detectable on other pancreatic LN DC subsets or on islet DC subsets (Fig. 4B; data not shown). Therefore, under a steady state, migratory CD103+ DC were responsible for cross-presenting islet-derived peptides in the pancreatic LN and had the potential to cross-prime CD8+ T cells. To directly examine the ability of the migratory CD103+ DC in stimulating CD8+ T cells, naïve OT1 cells were cocultured with LN CD103+ DC. Because the absolute number of the migratory DC in pancreatic LN was very low, we took advantage of the ability of FLT3L to expand these cells in vivo. The B16-FLT3L cell line produces FLT3L that drives in vivo expansion of FLT3L-dependent DC subsets and has no influence on the expression of costimulatory ligands or MHCI and MHCHI on any DC subset (38). B16-FLT3L cells (1 × 10⁶) were injected i.v. into C57BL/6 and RIPmOVA mice, and the DC were harvested after 18 d. FLT3L-expanded LN CD103+ DC from RIPmOVA but not from C57BL/6 mice stimulated OT1 cell to proliferate (Fig. 4C), demonstrating that migratory CD103+ DC were capable of cross-priming CD8+ T cells. CD103+CD11b+ DC showed greater ability for T cell stimulation than CD103+CD11b− DC (Fig. 4C).

To investigate whether DC subsets present Ag to CD4+ T cells, native OT2 cells expressing a transgenic TCR specific for OVA....
peptide presented by MHCII were labeled with CFSE and co-cultured with dispersed islet cells, pancreatic LN cells, or different DC subsets isolated from islets. Islet cells from RIPmOV A mice stimulated only ∼6% of OT2 cells to proliferate, whereas inflamed islet cells stimulated ∼16% of OT2 cells (Fig. 5A, 5B). Isolated DC subsets from uninflamed islets of RIPmOV A mice also poorly stimulated OT2 cells (Supplemental Fig. 1B). Pancreatic LN cells from RIPmOV A mice did not show significant differences in stimulating OT2 cells compared with pancreatic LN cells from C57BL/6 mice (Supplemental Fig. 1A). Taken together, these data suggest that islet and pancreatic draining LN DC poorly primed CD4+ T cells during the steady state. To amplify the T cell response, OT2 cells were stimulated with islet DC subsets pulsed either with OVA protein or MHCII-restricted OVA323–339 peptide. After 5 d of coculture, OVA protein-pulsed CD103+ DC stimulated OT2 cells to proliferate, whereas OVA-pulsed CD11b+ DC still did not (Fig. 5C). In contrast, only 50% CD11b+ DC pulsed with OVA peptide stimulated 50% of OT2 cells to proliferate (Fig. 5C, Supplemental Fig. 1B); thus, the inability of CD11b+ DC to stimulate CD4+ T cells was not a result of overall impairment in Ag presentation but was likely due to a limited ability to process Ag. Overall, islet CD11b+ DC had poor abilities to stimulate either CD8+ or CD4+ Ag-specific T cells under the steady-state condition.

The local microenvironment conditions DC and influences CD4 helper cell differentiation. To explore the influence of the local microenvironment of the islets on DC function, OT2 cells were cocultured with peptide-pulsed DC subsets. After 5 d, neither islet nor pancreatic LN DC subsets induced CD4+ T cells to express the Treg lineage marker Foxp3 in the absence of exogenous TGF-β added to the culture (Supplemental Fig. 2A). Flow cytometric analysis showed that peptide-pulsed islet CD103+ DC and CD11b+ DC were efficient in inducing OT2 cells to produce IFN-γ (10%), but inefficient for IL-4 (<0.5%) or IL-17 (<0.5%) production, indicating that neither subset under homeostatic conditions was capable of inducing Th2 or Th17 differentiation (Supplemental Fig. 2B). Overall, then, the islet DC poorly processed and presented Ag to CD4+ T cells, and the few T cells that were induced were of the Th1 lineage.

CD103+ DC have limited ability for Ag uptake

Tissue-resident DC constitutively phagocytose apoptotic cells and process and present peptides to T cells. To examine the ability of islet DC to sample and process islet-derived Ags, MIP-GFP mice were used. In this strain, GFP is expressed in the cytoplasm of β cells and can be taken up and processed by Ag presentation cells so that the phagocytosis of islet-derived Ags can be monitored by assaying the levels of green fluorescence in DC (26). During homeostatic conditions, ∼40% of islet CD11b+ DC and CD11b+ DC were efficient in inducing OT2 cells to produce IFN-γ (10%), but inefficient for IL-4 (<0.5%) or IL-17 (<0.5%) production, indicating that neither subset under homeostatic conditions was capable of inducing Th2 or Th17 differentiation (Supplemental Fig. 2B). Overall, then, the islet DC poorly processed and presented Ag to CD4+ T cells, and the few T cells that were induced were of the Th1 lineage.

FIGURE 3. CD103+ DC are the major migratory DC. (A) CCR7 expression on islet CD103+CD11b−/low and CD103+CD11b+ DC. Left panel, mRNA expression profile; pooled islet DC sorted from 10 mice. Right panel, Flow cytometry histogram. (B) Distribution of islet DC subsets by flow cytometry analysis in wild-type and CCR7−/− mice. Cells were gated on DAPI-CD45+MHCII+CD11c+ CD11b versus CD103. (C) Dot plots showing pancreatic LN DC subsets from wild-type and CCR7−/− and a histogram showing CX3CR1 expression from B6.CX3CR1GFP+ mice: gray solid line, B6.CX3CR1GFP+ islet CD11b+DC; black solid line, B6. CX3CR1GFP+ pancreatic LN CD103+CD11b+ DC; dashed line, B6.CX3CR1GFP+ pancreatic LN CD103+CD11b− DC; gray filled, B6 pancreatic LN CD103+CD11b+ DC. (D) Absolute numbers and percentages of CD11b+CD103−, CD103+CD11b−/low, and CD11b+CD103+ DC in pancreatic LN of C57BL/6 and CCR7−/−. n = 4–5. Mean ± SEM.

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progression of MLDS-induced inflammation. By day 3, MLDS treatment also caused slightly upregulation of CD86 and down-regulation of MHC II expression on CD11b+ DC but not on CD103+ DC (Fig. 6D), suggesting that resident islet CD11b+ DC acquired a more mature phenotype during islet inflammation. At this time, a marked decrease in both the percentage of GFP+ DC and the mean GFP fluorescence intensity were also observed in the CD11b+ DC subset, whereas the CD103+ DC subset remained GFP low (Fig. 6A, 6B). This suggested that internalized GFP Ag in CD11b+ DC had been processed and proteolytically destroyed during inflammation-induced maturation rather than recruitment of GFP+ DC from the circulation (26), whereas the ingested Ag remained largely intact and unprocessed under homeostatic conditions. No GFP+ migratory DC (CD8αα2CD11c+MHCII+) were found in either normal or MLDS-treated draining pancreatic LN (Fig. 6C). These data suggested that the low level of GFP signal in CD103+ DC may have been due to the limited capacity of these cells to sample islet-derived cell-bound Ags. Alternatively, those CD103+ DC that acquired tissue Ags may have migrated rapidly to the draining pancreatic LN and processed the ingested Ags during the migration, commensurate with ability of the migrated CD103+ DC to cross-present islet-derived Ag in the pancreatic LN.

We (Fig. 1A) and others (25) found that most islet DC were primarily located next to blood vessels and in contact with them and thus may have the capacity to sample Ag in the circulation. To address the ability of different islet DC subsets to take up soluble Ag, OVA-Alexa 488 was i.v. injected, and DC were examined for the presence of Alexa 488 signal 1.5 and 16 h later. As shown in Fig. 6E, ~25% of CD11b+ DC and relatively fewer CD103+ DC in both islets and pancreatic LN had green fluorescence, showing that although both DC subsets were able to sample soluble Ag from blood, CD11b+ DC were more efficient.

To rule out the possibility that this was caused by restricted access to sampling Ag from the blood by CD103+ DC, islet single-cell suspensions were incubated with OVA-Alexa 488 in vitro. Nearly 30% of CD11b+ DC took up OVA, but less than 10% of CD103+ DC did so. Uptake was an active process because it was inhibited by NaN3 and by incubation on ice (Fig. 6F, 6G). These data directly demonstrated that, as for cell-bound Ags, CD11b+ DC were more efficient than CD103+ DC in taking up soluble Ags.
the CD11b+ DC subset (Fig. 7A, 7B, 7D, 7E). Under inflammation induced by OT1 or OT2, CD11b+ DC slightly upregulated their surface MHCII and downregulated the surface MHCII, these CD11b+ DC were either macrophages and/or DC according to their function. Thus, it was likely that under a steady state, extracellular Ags captured by the CD11b+ DC were poorly processed in the endosome/phagosome of DC, and their degradation required phagosome maturation. Under islet inflammation, these resident CD11b+ DC underwent maturation as well, but there was no increase, suggesting other signaling was also involved, such as through TLRs, which have been shown to regulate Ag presentation (39–41). Although CD11b+ DC processed most of the internalized Ags following exposure to inflammatory stimuli, their costimulatory molecule expression did not markedly increase, suggesting other signaling was also involved, such as through TLRs, which have been shown to regulate Ag presentation (39–41). We and others showed that CD11b+ DC also have a limited ability to migrate to the draining LN (8, 11, 16, 43) where T cells are primed. Thus, CD11b+ DC populations may be more likely to modulate immune responses directly in the tissues by clearance of enteropathogens and/or dead cells by phagocytosis (8, 11, 16, 43) and thus may be more related to macrophages rather than DC according to their function.

In contrast, although the CD103+ DC had a limited capacity for sampling tissue or soluble Ag under the steady state, they were able to migrate to the pancreatic draining LN, stimulate CD4+ T cells, and cross-present tissue Ag to CD8+ T cells. It remains unknown what mechanisms account for the different abilities of CD103+ and CD11b+ DC in Ag presentation. The capability of splenic CD8+ DC to cross-present Ag seems dependent on the expression of specialized machinery for Ag processing (44). Peripheral CD103+ DC are developmentally related to CD8α+ DC (45). It will be interesting to investigate whether CD103+ DC also share similar specialized machinery with splenic CD8+ DC. The cross-presentation of self-Ag by DC in pancreatic LN has been demonstrated to induce CD8+ T cell tolerance (37, 46, 47). In vitro, we found that a few thousand LN CD103+ DC (DC/T cell: 1/16) from naive RIPmOVA hardly induced CD8+ T cells to proliferate (data not shown). Because of limited numbers of

**Discussion**

DC can be divided into subsets according to their development, phenotype, anatomic location, and function. The DC pool in islets consists mainly of two subsets: CD103+ DC and CD11b+ DC. The relative percentage of CD103+ DC was <20% of total islet DC and was much lower than the CD11b+ DC subset. We also noticed some individual variation in CD103+ DC, ranging from several percentages to 20% (7). Similar to origins of DC subsets in other nonlymphoid tissues (7–10), the development of islet CD103+ DC was dependent on Flt3 (7), whereas CD11b+ DC were mainly derived from monocytes.

DC are considered to be present as semimature cells with high phagocytic activity within peripheral tissues under homeostatic conditions (14, 16). We showed in this study that islet CD11b+ DC effectively took up islet Ag presumably derived from dead cells (26) and also sampled soluble Ags from blood. In contrast, CD103+ DC were in a more mature stage compared with CD11b+ DC under steady-state conditions and expressed higher levels of costimulatory molecules, lower levels of MHCII, and sampled less Ag (25). Although CD11b+ DC more efficiently took up Ags, the internalized Ags remained largely unprocessed under homeostatic conditions. In line with this, the CD11b+ DC poorly activated Ag-specific CD4+ or CD8+ T cells under homeostatic conditions. It has been shown that lamina propria CX3CR1+CD103+ DC also have poor T cell stimulatory capacity, although these DC effectively took up OVA in vivo (14, 16). Thus, it was likely that under a steady state, extracellular Ags captured by the CD11b+ DC were poorly processed in the endosome/phagosome of DC, and their degradation required phagosome maturation. Under islet inflammation, these resident CD11b+ DC underwent maturation as well as their subcellular compartments, which allows Ags stored in the endosome/phagosome to access contents of lysosomes and to be fully processed (39–41). Although CD11b+ DC processed most of the internalized Ags following exposure to inflammatory stimuli, their costimulatory molecule expression did not markedly increase, suggesting other signaling was also involved, such as through TLRs, which have been shown to regulate Ag presentation (39–42). We and others showed that CD11b+ DC also have a limited ability to migrate to the draining LN (8, 11, 16, 43) where T cells are primed. Thus, CD11b+ DC populations may be more likely to modulate immune responses directly in the tissues by clearance of enteropathogens and/or dead cells by phagocytosis (8, 11, 16, 43) and thus may be more related to macrophages rather than DC according to their function.

To characterize islet DC during Ag-specific inflammation, we induced T cell-mediated islet inflammation in RIPmOVA mice by the adoptive transfer of activated OT1 or OT2 cells. The phenotype of islet DC was examined 5 d after cell transfer. Both CD11c+ MHCII+ DC and large numbers of CD11c-MHCII− cells infiltrated into islets. A small percentage of these latter cells were CD11b+ or CD103+. Because these CD11c+ cells did not express surface MHCII, these CD11c+ cells were either macrophages and/or immature DC (Fig. 7A, 7D). Similar to MLDS-induced islet inflammation (Supplemental Fig. 3), CD4+ and CD8+ T cell-induced inflammation increased the number and percentage of the CD11b+ DC subset (Fig. 7A, 7B, 7D, 7E). Under inflammation induced by either OT1 or OT2, CD11b+ DC slightly upregulated CD86, CD40, and CD11b and downregulated surface MHCII expression (Fig. 7C, 7F). In contrast, the phenotype of CD103+ DC was unaffected by the Ag-specific CD8+ or CD4+ T cells (Fig. 7C, 7F), indicating that CD11b+ DC but not CD103+ DC became more mature in inflamed islets.

**Phenotype of islet DC during inflammation**

To characterize islet DC during Ag-specific inflammation, we induced T cell-mediated islet inflammation in RIPmOVA mice by the adoptive transfer of activated OT1 or OT2 cells. The phenotype of islet DC was examined 5 d after cell transfer. Both CD11c+ MHCII+ DC and large numbers of CD11c-MHCII− cells infiltrated into islets. A small percentage of these latter cells were CD11b+ or CD103+. Because these CD11c+ cells did not express surface MHCII, these CD11c+ cells were either macrophages and/or immature DC (Fig. 7A, 7D). Similar to MLDS-induced islet inflammation (Supplemental Fig. 3), CD4+ and CD8+ T cell-induced inflammation increased the number and percentage of the CD11b+ DC subset (Fig. 7A, 7B, 7D, 7E). Under inflammation induced by either OT1 or OT2, CD11b+ DC slightly upregulated CD86, CD40, and CD11b and downregulated surface MHCII expression (Fig. 7C, 7F). In contrast, the phenotype of CD103+ DC was unaffected by the Ag-specific CD8+ or CD4+ T cells (Fig. 7C, 7F), indicating that CD11b+ DC but not CD103+ DC became more mature in inflamed islets.
migratory DC in pancreatic LN, we were unable to increase the DC/T cell ratio without using FLT3L to expand DC number. FLT3L-expanded LN DC stimulated CD8+ T cells in vitro, showing that these migratory DC had potential for cross-priming. Furthermore, total LN cells, which contained similar or even fewer numbers of DC, effectively induced CD8+ T cell proliferation, suggesting that signals from other LN cells promoted this cross-priming process. One study reported a subpopulation of DC in pancreatic LN, which was named merocytic DC, based on their capacity to present islet Ags to both CD8+ and CD4+ T cells. These DC were CD11c+CD11b+PDCA-1- and whose number was increased in NOD mice. Katz et al. (38, 48) showed that purified islet Ag-loaded merocytic DC from diabetic NOD mice were able to break peripheral T cell tolerance to β cells and induce rapid onset type 1 diabetes in young NOD mouse. This suggests that merocytic DC may be a subpopulation of CD103+ DC, because they are phenotypically and functionally similar.

Calderon et al. (25) showed that 56% of DC from pancreatic LN of insulin promoter hen egg lysozyme (HEL) and 90% of DC from membrane HEL mice contained peptide–MHCII complex when examined by a specific Ab recognizing the complex. In these HEL transgenic mice, high concentrations of HEL are expressed in β cells of the islet, and low concentrations of HEL are found in the general circulation, comparable to those of insulin (49, 50). Thus, LN DC most likely sampled soluble HEL Ag and/or Ag transported by migratory DC, because >50% of LN DC were HEL+, whereas only 15% of LN DC were migratory. Calderon et al. showed that dispersed islets from IP-HEL mice were slightly less effective in stimulating the T cell hybridoma 3A9 compared with inflamed islets (25). Because T cell hybridomas may be much more sensitive to Ag and easily activated compared with primary T cells, this could account for their observations, which are in contrast to ours where we observed that uninfamed islets very poorly presented Ag to and stimulated CD4+ T cells. In RIPmOVA mice, in which OVA is membrane bound, we observed that dispersed islets much less effective in stimulating primary OT2 cells, compared with inflamed islets. The limited capacity of these DC for OT2 cell stimulation was likely due to very few OVA peptide–MHCII complexes presented during homeostasis, because ingested Ag was mostly unprocessed in this major phagocytic and nonmigratory DC subset. These data are consistent with previous reports that OT2 mice expressing mOVA in islets remained diabetes free for at least 8 mo (51, 52).

The tissue microenvironment shapes resident DC and regulates their functions. Unlike in skin, gut, and lung, neither DC subset in islets induced Treg differentiation. In contrast, both islet DC subsets possessed the potential to induce Th1 differentiation under nonpolarizing conditions, indicating that islet resident DC have less tolerogenic potential compared with mucosal DC but instead have the propensity to induce Th1 responses that have been thought to be involved in autoimmune type 1 diabetes (53). This suggests that tolerance is maintained in islets by the poor processing and presentation ability of the CD11b+ DC under the steady state.

During the progression of insulitis induced by autoreactive T cells, the balance between the activities and the relative proportions of these two islet DC changed. CD11b+ DC became more frequent and matured. Studies have been shown that T cell infiltration only leads to a mild increase in the percentage of islet DC expressing Ki67 (26). This small increase of DC proliferation
could not account for the much greater increase in the total DC numbers in the islets (26). Furthermore, the increase in islet DC after MLDS treatment depends on bone marrow function (25). Thus, it seemed most likely that the increase in islet CD11b+ DC was mainly due to recruitment from the circulation rather than local expansion of DC. Compared with CD11b+ DC, CD103+ DC remained unchanged. Therefore, CD103+ and CD11b+ DC had different functional responses. The balance between the activities of these subsets may be an important aspect of immune regulation in islets. Overall, the subsets differed in development, phenotype, uptake, and processing of Ags and the ability to present MHC-I- or MHC-II-restricted Ags. These differences are important in mechanisms of how islet DC orchestrate islet immunity.

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Disclosures
The authors have no financial conflicts of interest.
Figure S1. Direct presentation of islet-derived antigen by islet DC. (A) CFSE labeled $1 \times 10^5$ OT2 cells were co-cultured with $10^5 - 5 \times 10^5$ pancreatic LN cells from RIPmOVA or C57BL/6 for 4 days, and proliferation by CFSE dilution assayed. Cells were gated on CD4$^+$Vα2$^+$. Data from 3 separate experiments, duplicate to quadruplicate. Mean ± SEM. (B) CD103$^+CD11b^{−/low}$ and CD103$^−CD11b^+DC$ sorted from islets, pancreatic LN or inguinal LN of RIPmOVA mice. Islet DC cells were gated on FSC-A vs. FSC-W, DAPI$^-$CD45$^+$MHCII$^+$CD11c$^+$ and CD11b vs. CD103. DC subsets co-cultured with CFSE labeled $5 \times 10^4$ OT2 cells and OT2 proliferation analyzed after 5 days. Data from 2 separate experiments, duplicate. Mean ± SEM.
Figure S2. Th differentiation by DC. CD103<sup>+</sup>CD11b<sup>−low</sup>, CD103<sup>−</sup>CD11b<sup>+</sup> and CD103<sup>−</sup>CD11b<sup>+</sup> DC sorted from islets, pancreatic LN or inguinal LN of C57BL/6 mice were pulsed with OVA<sub>323-339</sub>. Islet cells were gated on FSC-A vs. FSC-W, DAPI<sup>−</sup>CD45<sup>−</sup>MHCII<sup>−</sup>CD11c<sup>−</sup> and CD11b vs. CD103. LN cells were gated on FSC-A vs. FSC-W, DAPI<sup>−</sup>CD8<sup>−</sup>MHCII<sup>−</sup>CD11c<sup>−</sup> and CD11b vs. CD103. 1x10<sup>5</sup> OT2 cells co-cultured with 500 DC in complete medium plus 10 ng/ml IL-2 and/or 2 ng/ml TGFβ for 5 days. T cells analyzed for (A) Foxp3; or (B) IFNγ, IL-4 and IL-17. Data representative of 2 separate experiments.
Figure S3. Identification of islet DC subsets during MLDS induced inflammation.

Islet inflammation was induced by MLDS in C57BL/6. (A) Islet DC gated on FSC-W CD45⁺MHCII⁺CD11c⁺ and CD11b vs. CD103, 0 and 3 days after initial MLDS treatment. (B) CD11b⁺CD103⁻ and CD103⁺CD11b⁻ DC gated on FSC-W CD45⁺MHCII⁺CD11c⁺ and CD11b vs. CD103. Graphs show percentage of CD103⁺DC and CD11b⁺ DC in MHCII⁺CD11c⁺ islet DC 0, 3 and 7 days after initial MLDS treatment. Each data point corresponds to one mouse, n=7-17. Mean ± SEM.