CD4⁺CD25⁺ Regulatory T Cells Prevent Type 1 Diabetes Preceded by Dendritic Cell-Dominant Invasive Insulitis by Affecting Chemotaxis and Local Invasiveness of Dendritic Cells

Mi-Heon Lee, Wen-Hui Lee, Ivan Todorov and Chih-Pin Liu

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CD4⁺CD25⁺ Regulatory T Cells Prevent Type 1 Diabetes Preceded by Dendritic Cell-Dominant Invasive Insulitis by Affecting Chemotaxis and Local Invasiveness of Dendritic Cells

Mi-Heon Lee,* Wen-Hui Lee,* Ivan Todorov,† and Chih-Pin Liu*†

Development of type 1 diabetes (T1D) is preceded by invasive insulitis. Although CD4⁺CD25⁺ regulatory T cells (nTregs) induce tolerance that inhibits insulitis and T1D, the in vivo cellular mechanisms underlying this process remain largely unclear. Using an adoptive transfer model and noninvasive imaging-guided longitudinal analyses, we found nTreg depletion did not affect systemic trafficking and tissue localization of diabetogenic CD4⁺ BDC2.5 T (BDC) cells in recipient mice prior to development of T1D. In addition, neither the initial expansion/activation of BDC cells nor the number of CD11c⁺ or NK cells in islets and pancreatic lymph nodes were altered. Unexpectedly, our results showed nTreg depletion led to accelerated invasive insulitis dominated by CD11c⁺ dendritic cells (ISL-DCs), not BDC cells, which stayed in the islet periphery. Compared with control mice, the phenotype of ISL-DCs and their ability to stimulate BDC cells did not change during invasive insulitis development. However, ISL-DCs from nTreg-deficient recipient mice showed increased in vitro migration toward CCL19 and CCL21. These results demonstrated invasive insulitis dominated by DCs, not CD4⁺ T cells, preceded T1D onset in the absence of nTregs, and suggested a novel in vivo function of nTregs in T1D prevention by regulating local invasiveness of DCs into islets, at least partly, through regulation of DC chemotaxis toward CCL19/CCL21 produced by the islets. The Journal of Immunology, 2010, 185: 2493–2501.

Onset of type 1 diabetes (T1D) is preceded by islet inflammation (insulitis), characterized by accumulation of immune cells, including dendritic cells (DCs) and T cells. Insulitis includes both peri-insulitis and invasive insulitis; the former is a nondestructive process and may not always lead to development of T1D, whereas the latter leads to islet destruction and T1D (1, 2). Therefore, stimulation that triggers progression from peri- to invasive insulitis is a key step in the prediabetic stage, leading to islet destruction and T1D. However, the cellular mechanisms underlying the maintenance of peri-insulitis and how its steady-state is broken down, resulting in invasive insulitis, have not been fully elucidated.

Foxp3⁺CD4⁺CD25⁺ regulatory T cells (nTregs) play a critical role in immune tolerance induction and autoimmune disease prevention (3). Although a deficiency in nTregs may contribute to early onset of insulitis and T1D in both humans and mice, the presence of a sufficient number of nTregs can rebuild tolerance, thus inhibiting T1D (4–9). Despite these exciting findings, the cellular mechanisms underlying the in vivo protective effect of nTregs against development of insulitis and T1D are still incompletely understood. In addition to T and B cells, innate immune cells, such as DCs, are among the first populations of immune cells detectable in NOD mice islets and may also contribute to insulitis and T1D (10–14). However, the role of DCs in the development of insulitis and diabetes, and the cellular requirements/mechanisms regulating their migration and infiltration into islets, remain largely unclear. It is known that nTregs’ in vitro function is cell-contact-dependent (15). Further studies showed that nTregs interact directly with DCs instead of T cells, and are able to negatively regulate activation, maturation, and trafficking of DCs to inflamed tissues (16–21). These findings suggest nTregs exert their regulatory function by inducing tolerance through interaction with innate immune cells like DCs.

Diabetogenic BDC2.5 (BDC) cells induce an aggressive form of T1D (22, 23). Studies on BDC scurfy mice showed that nTregs exerted their regulatory effects within pancreatic lesions and that lack of nTregs resulted in accelerated insulitis, suggesting nTreg function in islets to prevent T1D (24). However, the mechanisms by which nTregs prevent invasive insulitis remain elusive. In this study, adoptive transfer model studies involving BDC cells were used to address these important questions. Our findings support a model in which nTregs exert their in vivo function, at least in part, by regulating the local invasiveness of DCs instead of T cells from the islet periphery into islets, possibly through control of DC chemotactic activity, leading to the prevention of T1D.

Materials and Methods

Mice

BDC TCR transgenic NOD mice (23) were obtained from Drs. Mathis and Benoist (Joslin Diabetes Center/Harvard Medical School, Boston, MA). BDC mice expressing a firefly luciferase transgene under a β-globin promoter control (lac-BDC mice) have been previously described (25), and

*Department of Immunology and †Department of Diabetes and Metabolic Diseases, Beckman Research Institute, City of Hope, Duarte, CA 91010

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Address correspondence and reprint requests to Dr. Chih-Pin Liu, Department of Immunology, Department of Diabetes and Metabolic Diseases, Beckman Research Institute, City of Hope, 1450 E. Duarte Road, Duarte, CA 91010-3000. E-mail address: cpliu@coh.org

Abbreviations used in this paper: BDC, diabetogenic BDC2.5; BLI, bioluminescence imaging; DC, dendritic cell; I, invasive insulinitis; ILN, inguinal lymph node; ISL-DC, islet-infiltrating DC; LIV, liver; LNG, lung; nTreg, regulatory T cell; P, peri-insulitis; PAN, pancreatic region; PLN, pancreatic lymph node; ROI, region of interest; T1D, type 1 diabetes; SPL, spleen.

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were backcrossed with NOD mice for nine or more generations. All ani-
mal were kept in a pathogen-free animal facility at City of Hope, and
procedures used in animal studies have been reviewed and approved by
institutional review committee

Preparation of CD4+ and CD4+CD25− T cells for adoptive transfer

Splenic CD4+ and CD4+CD25− T cells were isolated from luc-BDC or
BDC mice by negative selection using magnetic beads (Miltenyi Biotec,
Auburn, CA). Purity of isolated cells was confirmed by staining with anti-
CD4 and the tetAγ7pβ7 tetramer that stains BDC cells (26, 27), and was
typically >95% for both cell types. Isolated BDC cells were i.v. injected
into 8- to 9-wk-old female NOD/scid recipient mice.

Bioluminescence imaging of T cell trafficking and region of
interest analyses of bioluminescent signal

The procedures for bioluminescence imaging (BLI) analysis have been
previously described (25). Briefly, r-luciferin (150 mg/kg body weight)
was i.p. injected into recipient mice, followed by anesthesia using
isofluorane. Recipient animals were analyzed for detection of biolu-
minescent signals of the transferred cells using an IVIS100 imaging system
(Xenogen, Alameda, CA). BLIs, with 2 min acquisition time, were taken
using a CCD camera 15 min after luciferin injection. The bioluminescent
signal was detected by quantifying the emitted light from selected tissues
using the region of interest (ROI) analyses. The ROI for each target tissue
was drawn over the corresponding tissue and the total flux (photons/sec)
was calculated (28) to represent the bioluminescent signal. The ROI for
pancreas covered areas for both pancreas and pancreatic lymph nodes
(PLNs), due to their close proximity.

Single-cell preparation from tissues and FACS analyses

Single-cell suspensions were prepared in PBS as previously described (25),
from dissected tissues, and used for FACS analyses. In brief, tissues were
treated with 2 mg/ml of collagenase-D (Roche Diagnostics, Indianapolis,
IN). Islet-infiltrating cells (ISL-DCs) present in the pancreas were prepared
as previously described (25) and were used for FACS analyses of cell
surface marker expression on CD11c+ DCs. Briefly, PLNs were removed
and the pancreas was distended with collagenase P, followed by a histopa-
que gradient (Sigma-Aldrich, St. Louis, MO). For FACS analyses, Ab
stained cells were analyzed using FACS Calibur (Becton Dickinson,
San Jose, CA), and data were analyzed using FlowJo (TreeStar Software,
San Carlos, CA). For stimulation of Ag-specific T cells, CD11c+ DCs were
further purified using anti-CD11c microbeads (Miltenyi Biotec), according
to the manufacturer’s instructions.

Immunohistochemistry and histology

Freshly isolated pancreata were frozen in Optimal Cutting Temperature
compound (Sakura, Torrance, CA) and cut into 5-μm sections. Adjacent
tissue sections were stained with primary Abs: rat anti-mouse CD4, hamster
anti-mouse CD11c (BD Biosciences, San Jose, CA), or polyclonal guinea pig
anti-insulin (Dako Canada, Mississauga, Ontário, Canada), and followed
with secondary Abs: Texas Red-donkey anti-guinea pig IgG or anti-rat
IgG, or FITC-goat anti-hamster IgG (Jackson ImmunoResearch, West
Grove, PA). For H&E staining, freshly isolated pancreata were formalin-
fixed, paraffin-embedded, deparaffinized, rehydrated, and cut as 5-μm
slices for staining.

Chemotaxis and chemokine receptor expression of ISL-DCs

ISL-DCs were prepared from pancreas of both groups of recipient mice on
day 11 after cell transfer. An aliquot of ISL-DCs was used for FACS analyses
of chemokine receptor expression. A majority of the ISL-DCs was used for DC
isolation with anti-CD11c microbeads (Miltenyi Biotec), according to
the manufacturer’s instructions. Purified ISL-DCs were then used in chemotaxis
assays using the Chemotex System, according to the manufacturer’s instruc-
tions (NeuroProbe, Gaithersburg, MD). Briefly, DCs were placed (20,000
cells/25 μl) in a 5-μm pore-sized filter in wells containing 30 μl medium
with chemokines (1 μg/ml). The total number of cells that migrated through
the filter was determined by cell counts after incubation (37°C, 3 h). Wells
without chemokines were used for calculation of nonspecific cell migration.

Statistical analyses

Two-tailed and two-sample unequal variance Student t test was used for
data analysis. Data were expressed as mean ± SD, with p < 0.05 consid-
ered statistically significant.

Results

Depletion of nTregs causes expedited T1D without altering
trafficking and tissue localization of BDC cells

Although it is known that nTreg-deficient CD4+CD25− effector
T cells can induce accelerated invasive insulitis and aggressive
T1D, the cellular mechanisms underlying how CD4+CD25− nTregs
exert their regulatory effect at the ISL, and prevent inflammation that
destroys ISL cells, remain largely unclear. BDC cells were used as
a model system and CD4+ BDC cells, with or without CD25weak+ nTreg depletion, were adoptively transferred to NOD/scid mice to investigate how nTreg function in vivo to inhibit insulitis and to
identify nTreg immune cell targets. The purity of transferred BDC cells
was assessed pre- and postdepletion (Fig. 1A), with CD4+CD25−
cells representing a population of CD25weakFoxp3− nTreg-
depleted T cells. As expected, recipient mice transferred with
nTreg-depleted CD4+CD25− T cells (nTreg-deficient mice), but
not control nondepleted CD4+ T cells, developed accelerated dia-
etes (Fig. 1B). Potential cellular mechanisms for the accelerated de-
velopment of T1D in nTreg-deficient mice are explored below.

It is possible that nTregs may modulate early systemic trafficking of
BDC cells during the prediabetic stage and subsequently block
their trafficking and homing to ISL, thus inhibiting invasive
insulitis that causes T1D. In the absence of nTregs, CD4+CD25−
BDC cells would migrate more rapidly to and accumulate faster in
ISL. To address this hypothesis, we investigated whether BDC
cells showed an altered trafficking pattern in nTreg-deficient mice,
using a novel animal model for noninvasive BLI-guided real-time
analysis of T cell trafficking (25). Our previous results obtained from
the ROI analyses of BLI and FACS analyses showed that the
bioluminescent signal emitted from tissues was proportional to
the number of T cells present in each tissue (25).

To monitor trafficking of BDC cells longitudinally in animals,
luciferase-expressing (luc+) CD4+CD25− or CD4+ BDC cells were
transferred into NOD/scid mice. Trafficking and tissue localization of
luc+ BDC cells in the two cohorts was monitored until after re-
cipient mice developed T1D. BLI analyses results showed that
during the prediabetic stage, on and before day 5 after cell transfer,
no significant difference in BLI signal (total flux) was noted between
the two cohorts (Fig. 1C, 1D). An increased BLI signal was detected in
various tissues in nTreg-deficient, but not control mice, only at the
postdiabetic stage, after development of T1D, on or after day 7 (Fig.
1C, 1D). This could have been due to a local expansion of BDC cells
that migrated to and resided in these tissues. These results demon-
strated comparable trafficking and tissue localization of BDC cells
during the prediabetic stage in both cohorts of mice.

Depletion of nTregs promotes progression and severity of
insulitis without affecting BDC cell number and activation
status during the prediabetic stage

We then hypothesized that, prior to T1D onset, nTreg function locally
by suppressing either function/activation or ISL infiltration of
BDC cells. This hypothesis further predicts that nTreg depletion
causes accelerated disease by inducing invasive insulitis that
destroys ISL at an earlier time point than in control mice. Indeed,
on day 5, H&E staining of pancreas sections showed a majority of
ISL (∼80%) in nTreg-deficient mice developed invasive insulitis,
compared with ∼7% in control mice, which displayed mostly peri-
insulitis or intact ISL (Fig. 2A). The difference in severity of
insulitis was further exemplified in recipient mice on day 11 when
∼97% of ISL in nTreg-deficient mice displayed severe invasive
insulitis (Fig. 2B). In comparison, the severity and nature of insu-
litis in control mice on day 11 was comparable to that observed on
day 5.
We next examined the hypothesis that accelerated invasive insulitis in nTreg-deficient mice during the prediabetic stage was due to an altered early expansion, accumulation, and/or activation of BDC cells in PLNs or ISL. FACS analyses revealed no significant difference in numbers of BDC cells detected in ISL or PLNs of both cohorts on day 5 prior to T1D (Fig. 3A). An increased...
number of BDC cells was detected in ISL of nTreg-deficient mice only during the postdiabetic stage (day 11). These results also suggested that the increased BLI signals in the pancreas region on day 11, in nTreg-deficient mice (Fig. 1C,1D), resulted solely from pancreas, not PLNs, as comparable numbers of BDC cells were detected in PLNs in both cohorts of mice (Fig. 3A). Additional studies showed that expression of the activation marker CD69 on BDC cells was comparable in PLNs and ISL of both cohorts on days 5 and 11 (Fig. 3B), further suggesting nTreg depletion did not affect the activation status of BDC cells in recipient mice. These results suggested nTreg depletion promoted invasive insulitis without causing an altered expansion/accumulation or activation of BDC cells in PLNs and pancreas during the prediabetic stage.

Accelerated T1D is not associated with altered populations of CD11c+ cells and NK cells during the prediabetic stage

Our results suggested that a population of immune cells, other than BDC cells, might contribute locally to accelerated invasive insulitis and T1D in nTreg-deficient mice. DCs are among the first population of immune cells detectable in ISL of NOD mice at 3–4 wk of age, but their role during insulitis and T1D remains largely unclear (10, 12–14). Other studies have shown nTregs may form aggregates with DCs in vitro and function through interaction with DCs, without directly contacting T cells (18, 29, 30). In nTreg-ablated mice, an increased NK cell population was detected in the ISL, suggesting they may participate in the pathogenesis of T1D in NOD mice (31). In addition, although NK cells were not detectable in NOD/scid mouse spleen, NK activity could be induced after polynosinic-polycytidylic acid stimulation of splenic cells (32). Therefore, we investigated whether nTreg-deficient recipient mice contained an altered population of DCs and/or NK cells in ISL or PLNs during prediabetic stage.

FACS analysis was used to compare CD11c+ DCs or CD49b+/CD3− NK cell populations present in cells isolated from ISL and PLNs of both cohorts of recipient mice. The results showed no significant difference in the percentage of CD49b+/CD3− NK cells detected in ISL and PLNs on day 5 in recipient mice transferred with either CD4+ or CD4+CD25− BDC cells (Fig. 4A,4B). There was also no difference in CD49b+/CD3− NK cells detected in ISL and PLNs on day 11. However, a significantly increased number of CD11c+, but not CD4+ cells, was detected in the ISL of mice transferred with CD4+CD25− cells on day 11 after they had developed T1D (Fig. 4A, lower right-hand panel), suggesting that during the prediabetic stage, recruitment of CD11c+ DCs and CD49b+/CD3− NK cells to ISL was not altered in nTreg-deficient mice. In contrast, recruitment of DCs to ISL was increased after onset of diabetes in these mice.

Accelerated invasive insulitis in nTreg-deficient mice is predominated by CD11c+ DCs, not CD4+ T cells

It is possible that nTreg depletion may disrupt the steady-state of peri-insulitis leading to aggressive infiltration of immune cells from the ISL periphery into ISL, resulting in invasive insulitis. This would suggest that nTregs exert their function in the ISL periphery, by regulating ISL infiltration of other immune cells, and cellular components of invasive insulitis would be altered in comparison with those of peri-insulitis maintained by nTregs.
Immunohistochemistry of pancreatic sections was performed to further elucidate the cellular component of immune cells infiltrated ISL.

In control mice (CD4+), both CD4+ and CD11c+ cells were detected in the periphery of ISL on days 5 and 11 (Fig. 5A, 5B, upper panels). ISL were intact with a large number of insulin-producing cells. Unexpectedly, although CD4+ cells were also detected in ISL periphery in nTreg-deficient mice (CD4+CD25−), a dominant population of CD11c− cells aggressively infiltrated the ISL during the prediabetic stage on day 5 (Fig. 5A, bottom panels). Many of the infiltrated CD11c− cells were in close proximity to or overlapped with insulin-producing cells (Fig. 5A, white arrows and yellow staining). In addition, on day 11, insulin-producing cells in nTreg-deficient mice were nearly all destroyed and the ISL were aggressively infiltrated by CD11c− cells, not CD4+ cells, most of which stayed within the periphery of destroyed and DC-filled ISL (Fig. 5B).

Fig. 5C, 5D provide a quantitative comparison of ISL differentially infiltrated by CD4+ or CD11c+ cells in CD4+ versus CD4+CD25− mice. These data showed that, during the prediabetic stage on day 5, a comparable percentage of ISL in both cohorts of mice developed CD4+ cell-peri-insulitis (88.2 versus 86.2%; Fig. 5C). In comparison, 60% of ISL in nTreg-deficient (CD4+CD25−) mice developed CD11c+ cell-invasive insulitis, whereas 55% of ISL in control (CD4+) mice developed CD11c− cell-invasive insulitis (83.3 versus 92.3%; Fig. 5D). In addition, on day 11, CD4+ cells of both cohorts remained in the ISL periphery (88.2 versus 69.3%; Fig. 5D). In comparison, the percentage of CD11c− cell-invasive insulitis in nTreg-deficient mice increased to ~96%, whereas CD11c+ cells in ~82% of ISL in control mice still remained in the periphery (Fig. 5D). Taken together, these results support the conclusion that nTregs depletion leads to accelerated invasive insulitis dominated by CD11c+ DCs, not by CD4+ cells.

Depletion of nTregs does not lead to altered maturation of ISL-infiltrated DCs and their ability to activate Ag-specific T cells

Previous studies have shown a defective population of APCs, including DCs, in NOD mice (33–35). To examine whether ISL-infiltrated CD11c+ DCs (ISL-DCs) were still able to stimulate Ag-specific T cells in nTreg-deficient mice, we first analyzed their ability to stimulate in vitro proliferation of BDC cells. ISL-CD11c+ cells from both cohorts were cultured with BDC cells plus peptide p79, a highly active BDC cell-stimulating peptide (36). The results showed that ISL-CD11c+ cells from both cohorts stimulated BDC cells equally well (Fig. 6A), indicating their comparable ability to stimulate Ag-specific T cells.

It is also possible that nTregs modulate the expression of costimulatory molecules such as CD80 or CD86 (30). Therefore, we next examined whether ISL-DCs from these mice bore altered phenotypes. We found that ISL-DCs expressed similar levels of class II MHC and costimulatory molecules, including CD40, CD80, CD86, and ICOS-L (Fig. 6B). Furthermore, ISL-DCs from both recipient cohorts did not express CD8a, representing a population of myeloid DCs. Our data also showed that CD11c+ DCs from both groups expressed CD11b on the cell surface (Fig. 6B). In comparison, these CD11c+ DCs expressed neither CD103 nor DEC205. Therefore, the CD11c+ DCs present either in the peri-insulitis or invasive insulitis

**FIGURE 5.** Accelerated invasive insulitis in nTreg-deficient mice is predominated by CD11c+ DCs not CD4+ cells. A and B, Immunohistochemistry staining of pancreas. Adjacent frozen sections from pancreata from CD4+ or CD4+CD25− cell transferred mice on (A) day 5 or (B) day 11 were coimmunostained with anti-insulin/anti-CD4, or anti-insulin/anti-CD11c. Although control mice ISL remained intact on days 5 and 11, ISL of nTreg-deficient mice showed disrupted architecture on day 5 and were dominated by CD11c+, not CD4+ cells. White arrows, surviving insulin-producing cells in ISL of nTreg-deficient mice. C and D, Quantitative comparison of ISL differentially infiltrated by CD4+ or CD11c+ cells. Up to 30 ISL were evaluated for degree of insulitis with infiltration of CD11c+ cells or CD4+ cells on (C) day 5 and (D) day 11. Scale bar, 50 μm. Original magnification ×100 or ×200 (the larger scale bar).
in both groups of recipient mice displayed the same phenotype. These results indicated that the expedited invasive insulitis was not attributed to an altered maturation and phenotype of DCs present in the ISL. In addition, our results suggested that nTregs functioned in the periphery of ISL to prevent DC-dominated invasive insulitis without affecting their phenotype and maturation.

**ISL-DCs from nTreg-deficient mice showed increased in vitro migration toward CCL19 and CCL21, but not CXCL10**

It has been shown that ISL can secrete chemokines, including CCL19, CCL21, and CXCL10, which may play a critical role in recruiting immune cells, including DCs, to ISL, leading to the development of insulitis and T1D (37–43). To identify the potential mechanisms of action underlying the dominant ISL-infiltration of DCs in nTreg-deficient mice, we investigated whether their ISL-DCs showed an altered in vitro chemotaxis toward CCL19, CCL21, and CXCL10.

**FIGURE 6.** nTreg depletion does not lead to an altered maturation of ISL-DCs and their ability to stimulate Ag-specific T cells. ISL-infiltrating cells were prepared from pancreata of the recipient mice on day 11 and were kept on ice before being used for the indicated assays. A, ISL-DCs from both cohorts present antigenic peptide to stimulate BDC cells equally well. ISL-DCs from the ISL-infiltrating cells were purified using anti-CD11c microbeads (>90% purity) and irradiated (3000 rad). CD4+ BDC cells (1.5 × 10^6) were cultured with purified ISL-DCs (8 × 10^3) with or without 1 μg/ml p79 for 4 d. [3H]Thymidine (1 μCi/well) was added to cell culture during the last 18 h, prior to cell harvest. NS, not significant. B, Phenotypic staining of ISL-DCs. An aliquot of the ISL-infiltrating cells from pancreata were used for FACS analyses without further purification for CD11c+ cells. These cells were stained with anti-CD11c plus indicated Abs. Shaded peaks, isotype controls. Dotted lines, CD4+ mice. Solid lines, CD4+CD25+ mice. FACS analyses were electronically gated on CD11c+ cells.

**FIGURE 7.** nTreg depletion leads to enhanced migration of ISL-DCs toward CCL19 and CCL21 chemokines without altering CCR7 expression on DCs. A, Expression levels of CCR7 and CXCR3 on ISL-DCs. ISL-infiltrating cells prepared from pancreata of either CD4+ or CD4+CD25+ cell-transferred recipient mice were stained with anti-CD11b and anti-CD11c plus anti-CCR7 or anti-CXCR3 for FACS analyses. Cells expressing CD11b/CD11c were electronically gated for further analyses on their expression of CCR7 or CXCR3. B, Chemotaxis analyses of ISL-DCs toward CCL19, CCL21, and CXCL10. In addition to an aliquot of the cells used for staining in (A), the majority of the same population of ISL-infiltrating cells were used to purify ISL-DCs using anti-CD11c microbeads for chemotaxis assays. Migration of the purified ISL-DCs in response to chemokines was examined as described in the Materials and Methods section. Cells cultured without chemokines were used as controls. Values represent mean ± SD of the total number of cells that migrated across the transwell; n = 3, in triplicate.
is important to note that, compared with control mice, a significantly greater number of ISL-DCs isolated from nTreg-deficient mice migrated toward either CCL19 or CCL21 (Fig. 7B).

These results demonstrated that nTregs are able to negatively regulate the chemotaxis response of ISL-DCs toward CCL19 and CCL21. The lack of nTregs may result in an enhanced ISL-DC chemotaxis without altering chemokine receptor expression on DCs.

**Discussion**

The novel findings in this study suggest that ISL DCs contribute locally to rapid development of invasive insulitis and T1D in nTreg-deficient mice. Therefore, nTregs may mediate their disease protective effect by directly affecting DCs instead of CD4+ T cells in ISL, perhaps through regulation of their chemotaxis responses to ISL chemokines. In support of this hypothesis, our results showed that nTregs helped maintain a steady-state of peri-insulitis without further development of T1D in animals. However, during the prediabetic stage, nTreg deficiency resulted in accelerated progression from peri- to invasive insulitis dominated by CD11c+ DCs, rather than CD4+ T cells, without affecting their ability to stimulate Ag-specific T cells and their maturation status. In addition, accelerated invasive insulitis might not be due to recruitment of different subsets of DCs to ISL in the absence of nTregs, because DCs from ISL of both mouse groups displayed the same phenotype as CD103+DEC205+ and CD8α+ myeloid DCs. Instead, our studies showed that nTregs were able to negatively regulate chemotaxis of ISL-DCs toward CCL19 and CCL21, suggesting nTreg deficiency may lead to enhanced ISL infiltration of DCs perhaps due to an altered response to these chemokines. Altogether, these findings support a model in which nTregs exert their in vivo function in T1D prevention, at least in part through regulating the local invasiveness of DCs from the ISL periphery into ISL, eventually leading to overt T1D.

Our results provide unique insights into the relative roles of CD4+ diabetogenic T cells, such as BDC cells, and nTregs in the development of insulitis and T1D. Although peri-insulitis can progress as a nondestructive process without causing T1D (1, 2), the cellular mechanisms underlying the maintenance of steady-state peri-insulitis and its break down leading to invasive insulitis are largely unclear. Compared with nontransferred recipient mice, the presence of BDC cells was required for the development of invasive insulitis and diabetes. However, a majority of BDC cells remained in the ISL periphery, regardless of whether nTregs were present, suggesting they were not the primary effector cells participating directly in ISL destruction. Consistent with this hypothesis, depletion of nTregs increased insulitis severity without changing the activation status of BDC cells. This indirect role of BDC cells is also supported by studies showing that CD4+ T cells remained in ISL periphery in diabetic NOD mice (44), and that BDC cells may not function as direct effectors in ISL cell destruction in BDC mice (45, 46).

Although the current study does not address the role of BDC cells, it is possible they may promote the trafficking of other immune cells, such as DCs, to PLNs and ISL. In the absence of nTregs, BDC cells may further promote ISL infiltration of DCs, leading to ISL destruction. Alternatively, in the presence of pathogenic BDC cells, primed DCs in the ISL of nTreg-deficient mice may be more effective in activating BDC cells, further contributing to the subsequent destruction of ISL. The presence of nTregs may lead to suppression of these processes. However, we consider this possibility less likely because the activation status of BDC cells was not altered and the DCs in ISL of both groups of recipient mice were able to present Ag to stimulate BDC cells equally well (Figs. 3B, 6A). Therefore, although T1D has been considered a T cell-mediated autoimmune disease and diabetogenic CD4+ T cells are required, they may not directly infiltrate ISL and contribute to ISL cell destruction. Further studies are necessary to examine whether these findings can also be applied to other types of diabetogenic T cells.

It has been previously shown that nTregs can inhibit NK cells both in vitro and in vivo (47), and they can control NK cell homeostasis in lymph nodes (48). Temporal ablation of nTregs in NOD mice caused accumulation of NK cells and enhanced NK cell activities, suggesting an important role of NK cells in T1D (31). Although NOD/scid mice have a barely detectable NK cell population, NK activity could be induced after polyinosinic-polycytidylic acid stimulation (32). Our results showed that a small and comparable population of CD3+CD49b+ cells was detected in ISL and PLNs in both cohorts of mice (Fig. 4B), suggesting that, if they were involved in T1D pathogenesis in these mice, they may not be the major effector population. However, these results do not exclude the possibility that these NK cells may interact with and affect DCs during the increased ISL infiltration of DCs.

Our findings also demonstrated that nTregs can exert their in vivo function in ISL, instead of in PLNs, at least in part through suppressing the development of myeloid DC-mediated invasive insulitis. Previous studies provided evidence that CD11c+CD8α− myeloid DCs may contribute to insulitis, resulting in T1D (13). Our results further showed that DCs present in ISL of either control mice with dominant peri-insulitis or nTreg-deficient mice with dominant invasive insulitis were CD11c+CD8α− myeloid DCs. Therefore, induction of differential DC-mediated insulitis in these recipient mice was not due to recruitment of varied subsets of DCs, but to differences in the ability of resident myeloid DCs to infiltrate ISL.

Previous reports showed that nTregs can negatively regulate activation, maturation, and trafficking of DCs (16, 17, 20, 21), and form aggregates on DCs in vitro (30). In contrast, effector T cell-mediated inflammation may induce maturation of ISL-resident DCs (49), suggesting active functional interaction between DCs and different T cell populations. In our studies to examine how nTreg deficiency may cause DC-dominated invasive insulitis, we found that the accelerated DC-dominated invasive insulitis in nTreg-deficient recipient mice was not due to an altered ability of DCs to activate Ag-specific T cells and maturation of DCs. However, although our results showed ISL-DCs from both groups of recipient mice were able to equally stimulate BDC cells in response to p79, there was no need for DCs to process p79 intracellularly to present it to BDC cells, as it would for a protein. Therefore, these results do not exclude the possibility that DCs from these two groups of mice may differ in their ability to process protein Ags in stimulating target T cells. Nevertheless, our further mechanistic studies showed that nTreg deficiency resulted in enhanced DC chemotaxis toward CCL19/CCL21 without affecting chemokine receptor expression on ISL DCs. These results suggest that the enhanced chemotaxis is not due to an altered expression of chemokine receptors for these chemokines. Although the exact mechanisms underlying this observation remain unclear, previous studies have also shown that DCs expressing the same levels of chemokine receptors could migrate differently toward their ligand. For example, a recent study provided evidence that Jak3-deficient but not wild-type DCs showed impaired chemotaxis toward CCL19 and CCL21, although these DCs expressed comparable levels of CCR7 (50). These results suggested that Jak3 may control chemotaxis of DCs toward CCL19/CCL21. Therefore, it is possible that the enhanced ISL-DC migration in nTreg-deficient mice in our studies was due to altered intracellular signaling involving molecules such as Jak3. In addition, it is also possible that ISL-DCs in recipient mice at different time points prior...
to diabetes onset may express varied levels of CCR7 that lead to differential migration of ISL-DCs toward chemokines. Further studies are necessary to address such possibilities.

In summary, the current findings demonstrate that nTregs may function locally in ISL to prevent inflammatory responses that lead to invasive ISL infiltration by DCs. Our results further suggest that nTregs may do so, at least in part, through regulating chemotaxis of DCs toward CCL19 and CCL21. Our data demonstrate DC-dominated invasive insults preceded TID onset and suggest a novel in vivo function of nTregs in preventing the disease by regulating local invasiveness of DCs, not CD4+ T cells, into ISL. In the absence of nTregs, pathogenic CD4+ T cells in the neighboring ISL periphery area may help DCs to more aggressively infiltrate ISL, resulting in their destruction. Further studies are necessary to examine whether ISL-infiltrated DCs in nTreg-deficient mice are directly involved in ISL cell destruction. Overall, these studies provide novel insights into the pathogenesis of TID and suggest an alternative mechanism underlying the in vivo action of nTregs in preventing insulitis, with new potential opportunities for therapeutic strategies in the treatment of TID.

Disclosures
The authors have no financial conflicts of interest.

References


