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Cutting Edge

Cutting Edge: Origins, Recruitment, and Regulation of CD11c+ Cells in Inflamed Islets of Autoimmune Diabetes Mice

Joanna E. Klementowicz,* Ashely E. Mahne,* Allyson Spence,* Vinh Nguyen,* Ansuman T. Satpathy,† Kenneth M. Murphy,‡ and Qizhi Tang*

In NOD mice, CD11c+ cells increase greatly with islet inflammation and contribute to autoimmune destruction of pancreatic β cells. In this study, we investigated their origin and mechanism of recruitment. CD11c+ cells in inflamed islets resembled classical dendritic cells based on their transcriptional profile. However, the majority of these cells were not from the Zbtb46-dependent dendritic-cell lineage. Instead, monocyte precursors could give rise to CD11c+ cells in inflamed islets. Chemokines Ccl5 and Ccl8 were persistently elevated in inflamed islets and the influx of CD11c+ cells was partially dependent on their receptor Ccr5. Treatment with islet Ag-specific regulatory T cells led to a marked decrease of Ccl5 and Ccl8, and a reduction of monocyte recruitment. These results implicate a monocytic origin of CD11c+ cells in inflamed islets and suggest that therapeutic regulatory T cells directly or indirectly regulate their influx by altering the chemotactic milieu in the islets. The Journal of Immunology, 2017, 199: 000-000.

Most non-lymphoid tissue dendritic cells (DCs) can be divided into CD103+ and CD11b+ subsets. The CD103+ subset arises primarily from bone marrow pre-DC precursors, whereas the CD11b+ subset can arise from either pre-DCs or monocytes (1, 2). The heterogeneity of non-lymphoid tissue CD11b+ DCs is reflected in their transcriptional profile and the distinct functions they serve in the tissue (3).

In normal islets, the predominant immune cells are CD11c+ CD11b− cells that originate from CSF1-dependent monocyte precursors (1, 4–6). CD11c+ cells in the NOD mouse pancreas have a distinct gene expression profile and their number greatly increases with inflammation in the islets (7, 8). Depletion of phagocytes or CD11c+ cells resists islet inflammation and delays diabetes development (9, 10), demonstrating the importance of these cells in type 1 diabetes pathogenesis. However, more recent studies suggest the existence of different subsets of myeloid cells in inflamed islets that serve distinct functions. For example, a subset of Crlg-expressing myeloid cells are proposed to be protective against diabetes progression (11) and a rare subset of Batf3-dependent CD11b+CD103+ DC are essential for disease initiation in NOD mice (12). The origin and identity of the majority of CD11c+ cells in inflamed islets remains unknown, however, and is the focus of this study.

Materials and Methods

Mice

NOD.Rag2−/−, NOD.CD28−/−, NOD.CD11c-YFP.CD28−/−, NOD.BDC2.5 Thy1.1 TCR transgenic, B6, B6.Zbtb-GFP, B6.Ccr2−/−, B6.Ccr5−/−, B6.Ccr7−/−, and B6.Cx3cr1-GFP mice were housed and bred under specific pathogen-free conditions at the University of California, San Francisco Animal Barrier Facility. The Institutional Animal Care and Use Committee approved all experiments.

Flow cytometry of islet infiltrates

Islets were isolated and dissociated as previously described (13). Peritoneal cells were collected by lavage. Splenocytes were prepared with 250 μg DNase I and 800 Mani Ul/ml collagenase D (Roche). Cells were stained with Abs to CD45 (30-F11), Ly5.1 (A20), Ly5.2 (104), B220 (RA3-3A1/6.1), CD11c (N418), CD11b (M1/70), CD103 (2E7), F4/80 (BM8), CD115 (AFS98), Sirpa (P84), DCIR2(33D1), and Ly6c (HK1.4). DAPl (Invitrogen) was used to exclude dead cells. Cells were analyzed and purified using a BD FACS Aria II or Fortessa.

Quantitative real-time PCR

mRNA from purified cells or whole islets was extracted using Arcturus Pico Kit (Life Technologies). Reverse transcription was done using an 5′Ct Advanced cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using SYBR Green Master Mix (Bio-Rad) and primers from Qiagen and Bio-Rad on a Bio-Rad CFX 96.

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The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional DC; DC, dendritic cell; 5-FU, 5-fluorouracil; Teff, effector T cell; Treg, regulatory T cell.
Chemokine protein measurements

Handpicked islets were cultured overnight at 37°C in RPMI 1640 with 10% FCS at a density of 2 ml per islet. Chemokines in the supernatants or cell lysate including the supernatant were measured using ELISA (CCL8 from Bosterbio and CCL7 from Thermo Fisher) or multiplex Luminex (all other chemokines; Eve Technologies).

Monocyte trafficking

Monocytes were isolated from NOD.Rag2−/− bone marrow using an EasySep Mouse Monocyte Isolation Kit (Stemcell). The cells were labeled with 5 μM CSFE and were retro-orbitally injected into recipient mice. In vivo monocyte labeling was achieved by retro-orbital injection of 1 μm fluorescent beads (Polysciences) as previously described (14).

5-fluorouracil chimeras

Mice were injected i.p. with 150 mg/kg of 5-fluorouracil (5-FU) (Sigma) in sterile PBS a day before transfer of T cell depleted bone marrow.

Enumerating islet CD11c+ cells

Handpicked islets from NOD.CD11c-YFP mice were stained in 10 μM CMTMR and embedded in RPMI 1640 medium with 0.5% agarose (Invitrogen) on a coverslip. Images of islets were acquired on a custom-built 4-PMT-detector video-rate two-photon microscope using a water immersion 20×/0.95 NA objective with the aid of Micromanager Software. Data were analyzed using Imaris (Bitplane).

T cell transfers

FACS-purified CD4+CD62L+CD25+ regulatory T cells (Tregs) and CD4+CD62L+CD25− effector T cells (Teffs) from the lymph nodes of NOD. BDC2.5.Thy1.1 TCR transgenic mice were expanded as previously described (15). BDC2.5 Treg treatments were administered via i.p. injection into NOD.CD28−/− mice (106 cells) at 5–7 wk of age. Then 106 BDC2.5 Teff were i.p. injected into NOD.Rag2−/− mice to induce islet inflammation.

Statistical analysis

Statistical analyses were performed with the aid of Prism software (GraphPad).

Results and Discussion

CD11c+CD11b+ cells in inflamed islets have a conventional DC transcriptional signature

Autoimmune-mediated inflammation in the pancreatic islet leads to a massive influx of CD11c+CD11b+ cells (13). A previous study found a minor population of Batf3-dependent CD103+ DCs in the inflamed islets (12). However, the identity of the majority of the CD11c+ cells remains to be defined. We have examined islet CD11c+CD11b+ cells in NOD.CD28−/− mice that develop diabetes with higher penetrance and better synchrony than wild-type NOD mice due to a deficit in Tregs (16). Similarly to NOD mice (5), the majority of CD11c+ cells in inflamed islets of NOD.CD28−/− mice were a fairly uniform population of CD11b+F4/80+CD103−Sirpa+, and dim for CD4 and DCIR2 (Fig. 1A, Supplemental Fig. 1). Thus, these cells do not resemble lymphoid tissue CD11c+CD11b+ conventional DC (cDC2) but are more similar to monocyte-derived DCs (17).

Studies from the ImmGen consortium have revealed that in some nonlymphoid tissues, cells previously described as DCs are transcriptionally more closely related to macrophages than...
to DCs. By profiling expression levels of both cDC and macrophage signature genes in inflamed islet CD11c+ cells, we sought to more definitively determine the identity of these cells. We profiled the expression of cDC and macrophage signature genes defined by the ImmGen consortium (3, 18) in CD103+ and CD103− CD11c+ cells in inflamed islets. Both subsets expressed core cDC genes (Fig. 1B) and lacked the expression of macrophage signature genes except Fc receptor, IgG and high affinity I (Fig. 1C). We quantitatively analyzed the level of “cDC-ness” and “macrophage-ness” for each cell type by calculating the average relative expression for all DC signature genes and all macrophage signature genes, respectively. The CD11c+CD11b+ splenic DCs were given a cDC score of 1 and a macrophage score of 0; whereas the peritoneal macrophages were assigned a cDC score of 0 and a macrophage score of 1. CD11c+CD11b+ cells in inflamed islets showed a stronger alliance with cDCs than with macrophages, with the CD103+ subset demonstrating stronger cDC-ness than the CD103− subset (Fig. 1D). Thus, at the transcriptional level, CD11c+CD11b+ cells in inflamed islets possessed a DC-like phenotype.

Monocytes, but not pre-DCs, contribute to CD11c+CD11b+ cells in inflamed islets

We next sought to determine the precursor of CD11c+ cells in inflamed islets. To this end, we developed a 5-FU bone marrow chimera approach, in which Ly5.1+ NOD.Rag2−/− mice were treated with 5-FU to ablate their bone marrow 1 d before receiving Ly5.2+ bone marrow cells as a source of DC precursors. The use of 5-FU instead of radiation preserves endogenous CD11c+ cells and architecture of lymphoid organs, thus minimizing impacting peripheral immune status and diabetes onset (Supplemental Fig. 2A). One week after bone marrow reconstitution, some mice were injected with islet Ag–specific CD4+CD25− Teff from BDC2.5 TCR transgenic mice to induce islet inflammation (Supplemental Fig. 2B). Ly5.2+ cells appear in the blood 1 wk after bone marrow cell injection and can be found among CD11c+ MHCIId+ cells in the islets at 2 wk (Supplemental Fig. 2C). Transfer of BDC2.5 Teff resulted in a significant increase of Ly5.2+ cells in the islets, demonstrating that bone marrow could give rise to islet CD11c+ cells during inflammation (Supplemental Fig. 2D, 2E).

We then used this model to define the origin of CD11c+ cells in inflamed islets. Zbtb46 distinguishes cDCs from other mononuclear phagocytes, and the Zbtb46-GFP reporter mouse is a valuable tool for identifying cDCs. We reconstituted 5-FU–treated Ly5.1+ NOD.Rag2−/− mice with bone marrow cells from Ly5.2+ Zbtb46-GFP reporter mice and found more than 80% of Ly5.2+ CD11c+CD11b+ cells did not express GFP (Fig. 2A). In comparison, most of the Ly5.2+CD11c+ cells in the spleen and draining pancreatic lymph nodes were GFP+ (Fig. 2A). Although we have not transferred pre-DC to further validate our findings, these data suggest that the majority of CD11c+ cells in inflamed islets do not arise from pre-DC precursors, despite their DC-like gene expression profile.

Steady-state islet CD11c+ cells have a monocytic origin (5). To determine whether CD11c+ cells in inflamed islets share this origin, we transferred CFSE-labeled bone marrow CD115+ monocytes to NOD.Rag2−/− mice that had received BDC2.5 Teff. CFSE+ cells were readily detectable in inflamed islets 36 h after monocyte transfer and the majority of these cells were CD11c+CD11b+CD103− (Fig. 2B and data not shown). These findings show that monocytes are recruited to inflamed islets and can give rise to islet CD11c+ cells.

**FIGURE 2.** Monocyte contribution to the islet CD11c+ cell population. (A) 5-FU chimera mice were generated in Ly5.1+ NOD.Rag2−/− recipients using Ly5.2+ B6.Zbtb46-GFP bone marrow. Representative flow plots show the profiles of Ly5.2+ donor-derived islet-infiltrating cells (top left) and their expression of Zbtb46-GFP. Summary of the percentages of GFP+ cells in various populations of Ly5.2+ cells (bottom right). Each symbol represents one mouse and bars represent mean + SEM. Data are a summary of two independent experiments. (B) Flow cytometric analysis of adoptively transferred monocytes in inflamed islets. Representative flow plots along with quantification of CD11b+ or CD11b− islet cells among CFSE+ transferred cells (bottom right) are shown. Each symbol represents one mouse and bars represent mean + SEM. Data are a summary of two independent experiments of two mice per experiment. Statistical analysis was performed using one-way ANOVA (A) or Student t test (B). **p < 0.01, ***p < 0.001, ****p < 0.0001.
Islet CD11c+ cell recruitment is partially dependent on Ccr5

Monocyte recruitment to inflamed tissues is mainly mediated through the chemokine receptor Ccr2, and roles for Ccr1 and Ccr5 have been implicated (19). To determine the chemokine ligand-receptor pair(s) involved in the recruitment of CD11c+ cells to inflamed islets, we surveyed the expression of CC chemokines in inflamed islets of prediabetic NOD.CD28−/− mice and compared with those from non-inflamed islets in NOD.Rag2−/− mice. Although myeloid cells in NOD.Rag2−/− mice may have altered functions due to their development in the absence of adaptive immune cells (20), islets from NOD.Rag2−/− have a similar transcriptional profile, including chemokine expression, to young NOD mice before the onset of inflammation (21).

Ccl5 and Ccl8 were elevated at multiple ages in NOD.CD28−/− islets when compared with NOD.Rag2−/− controls (Fig. 3A). In addition, Ccl5 and Ccl8 protein were upregulated in culture supernatants of NOD.CD28−/− islets when compared with NOD.Rag2−/− islets (Fig. 3B).

To determine the requirement of specific chemokine receptors for recruiting CD11c+ cells to inflamed islets, we injected 5-FU–treated NOD.Rag2−/− mice with a 50:50 mixture of wild-type and chemokine receptor–deficient bone marrow cells (Fig. 3C) and tracked their migration to inflamed islets after...
BDC2.5 Teff transfer. The chemokine receptors tested included Ccr2, Ccr5, Ccr7, and Cx3cr1, all of which have been previously implicated in monocyte and DC recruitment.

Ccr2 is preferentially expressed by Ly6c<sup>hi</sup> monocytes and has been shown to mediate recruitment of monocyte-derived DC to inflamed colon and lung by binding to Ccl2 (19). However, we found that Ccr2<sup>−/−</sup> cells had no deficiency in entering inflamed islets (Fig. 3D). Similarly, Ccr7 was not required for CD11c<sup>+</sup> cell recruitment into inflamed islets. Cx3cr1 is expressed on Ly6c<sup>lo</sup> patrolling monocytes in the blood and CD11c<sup>+</sup> cells in non-inflamed islets (5). In our experiments, Cx3cr1<sup>−/−</sup> cells were more efficiently recruited into the inflamed islets than wild-type cells (Fig. 3D).

Previous data on the role of Ccr5 in the pathogenesis of diabetes in NOD mice are mixed. Anti-Ccr5 Ab reduces insulin and delays diabetes (22) whereas Ccr5-deficient NOD show accelerated diabetes (23). Ccr5 is the receptor for Ccl5 and Ccl8 (24), two chemokines persistently elevated in inflamed islets. In mice that received a mixture of wild-type and Ccr5<sup>−/−</sup> bone marrow, we found higher proportions of Ccr5<sup>−/−</sup> cells among blood Ly6c<sup>lo</sup> monocytes but the same contribution of wild-type and Ccr5<sup>−/−</sup> cells to other blood leukocyte populations, suggesting a selective buildup of Ccr5<sup>−/−</sup> Ly6c<sup>lo</sup> monocytes in the blood (data not shown). Despite this, wild-type cells preferentially gave rise to CD11c<sup>+</sup> cells in the inflamed islets. Thus, Ccr5<sup>−/−</sup> cells were at a significant disadvantage to give rise to CD11c<sup>+</sup> cells in the inflamed islets (Fig. 3D). It is noteworthy that some Ccr5<sup>−/−</sup> cells entered the inflamed islets, suggesting redundant mechanisms or partial compensation for this process. Ccr1 can also bind to Ccl5 and Ccl8 (25) and may explain the mixed results of the role of Ccr5 in previous reports and the partial effects we observed in this study. Altogether, these data suggest a role for Ccr5, but not Ccr2, Ccr7, or Cx3cr1 in the recruitment of CD11c<sup>+</sup> cells to inflamed islets. It is important to note that these bone marrow chimera experiments used B6 donors that lack the intrinsic NOD autoimmune predisposition, and NOD pancreatic CD11c<sup>+</sup> cells were reported to have lower expression of Ccr5 (7); thus, validation using donors on the NOD background is warranted.

**Islet Ag-specific Treg attenuate CD11c<sup>+</sup> cell recruitment to inflamed islets**

Adoptive transfer of BDC2.5 Tregs protects against diabetes development, both in NOD and NOD.CD28<sup>−/−</sup> mice (15, 26). We next determined the impact of Treg therapy on the accumulation of CD11c<sup>+</sup> cells in inflamed islets. Treg treatment suppressed Ccl2, Ccl5, Ccl7, Ccl8, and Ccl9 mRNA expression in inflamed islets (Fig. 4A). Of these, Ccl5 showed the most dramatic reduction (84%), followed by Ccl8 (78%). When examined at the protein level, lysates from islets of Treg-treated NOD.CD28<sup>−/−</sup> mice had less Ccl5 than those from islets of age-matched controls, whereas Ccl2 was unchanged (4B). Moreover, Treg treatment led to a significant reduction in islet CD11c<sup>+</sup> cell numbers when compared with untreated age-matched controls (Fig. 4C, 4D).

To determine if reduction of islet CD11c<sup>+</sup> cells was due to decreased islet CD11c<sup>+</sup> cell recruitment after Treg treatment, we used an in vivo monocyte-labeling method to track the recruitment of monocyte precursors to the islets (14). Fluorescently labeled latex beads were injected directly into the bloodstream of Treg-treated or age-matched NOD.CD28<sup>−/−</sup> mice. The beads were taken up by blood monocytes (Fig. 4E), thus providing a means to track their trafficking into inflamed islets. In mice that received BDC2.5 Tregs 2 wk prior to bead injection, significantly fewer bead<sup>+</sup> CD11c<sup>+</sup> cells were detected in islets (Fig. 4F). Together, these findings show that Treg treatment leads to a reduction in inflammatory chemokines,
decreased recruitment of CD11c+ cells, and reduced CD11c+ cell accumulation in inflamed islets. Previously we have shown Treg therapy rapidly suppresses IFN-γ production by intraislet CD4+ and CD8+ T cells within days (27). By comparison, the effect of Tregs on CD11c+ cells was more gradual and took 1–2 wk to manifest (data not shown). Inhibition of IFN-γ production may be an upstream event that leads to downregulation of inflammatory chemokines in the islets, as Cd5 can be regulated by IFN-γ (28).

In summary, this work demonstrates in a model of autoimmune diabetes that CD11c+ cells in inflamed islets were mostly derived from monocytes despite their DC-like phenotype. These cells were attracted to the inflamed islets in part via Ccr5, likely in response to elevated Ccl5 and Ccl8. Therapeutic Tregs decreased Ccl5 and Ccl8 expression and CD11c+ cell recruitment to the islets, pointing to the possible role of this process in propagating autoimmune pathology.

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Disclosures
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