

Structural Changes of Cutaneous Immune Cells in Patients With Type 1 Diabetes and Their Relationship With Diabetic Polyneuropathy

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Abstract

Background and Objectives

Diabetic polyneuropathy (DPN) is a complication of diabetes characterized by pain or lack of peripheral sensation, but the underlying mechanisms are not yet fully understood. Recent evidence showed increased cutaneous macrophage infiltration in patients with type 2 diabetes and painful DPN, and this study aimed to understand whether the same applies to type 1 diabetes.

Methods

The study included 104 participants: 26 healthy controls and 78 participants with type 1 diabetes (participants without DPN [$n = 24$], participants with painless DPN [$n = 29$], and participants with painful DPN [$n = 25$]). Two immune cells, dermal IBA1⁺ macrophages and epidermal Langerhans cells (LCs, CD207⁺), were visualized and quantified using immunohistological labeling and stereological counting methods on skin biopsies from the participants. The IBA1⁺ macrophage infiltration, LC number density, LC soma cross-sectional area, and LC processes were measured in this study.

Results

Significant difference in IBA1⁺ macrophage expression was seen between the groups ($p = 0.003$), with lower expression of IBA1 in participants with DPN. No differences in LC morphologies (LC number density, soma cross-sectional area, and process level) were found between the groups (all $p > 0.05$). In addition, IBA1⁺ macrophages, but not LCs, correlated with intraepidermal nerve fiber density, Michigan neuropathy symptom inventory, (questionnaire and total score), severity of neuropathy as assessed by the Toronto clinical neuropathy score, and vibration detection threshold in the whole study cohort.

Discussion

This study showed expressional differences of cutaneous IBA1⁺ macrophages but not LC in participants with type 1 diabetes-induced DPN compared with those in controls. The study suggests that a reduction in macrophages may play a role in the development and progression of autoimmune-induced diabetic neuropathy.

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Glossary

BMI = body mass index; **CDT** = cold detection threshold; **Dia BP** = diastolic blood pressure; **DPN** = diabetic polyneuropathy; **eGFR** = estimated glomerular filtration rate; **HDL** = high-density lipoprotein; **IBA1** = ionized calcium binding adaptor molecule 1; **IENFD** = intraepidermal nerve fiber density; **IQR** = interquartile range; **LC** = Langerhans cell; **LDL** = low-density lipoprotein; **MHC** = major histocompatibility complex; **MNSI** = Michigan Neuropathy Screening Instrument; **MNSI-Q** = Michigan Neuropathy Screening Instrument questionnaire; **NRS** = numeric rating scale; **P-T1DPN** = type 1 diabetes with painful diabetic polyneuropathy; **ROI** = region of interest; **Sys BP** = systolic blood pressure; **TCNS** = Toronto clinical neuropathy score; **T1D** = type 1 diabetes without diabetic polyneuropathy; **T1DPN** = type 1 diabetes with painless diabetic polyneuropathy; **WDT** = warm detection threshold.

Introduction

Approximately half of people with diabetes eventually develop diabetic polyneuropathy (DPN), which usually presents in a “stocking and glove distribution”.¹ Cutaneous small nerve fibers are unmyelinated or thinly myelinated, making them vulnerable to damage due to hyperglycemia-induced tissue inflammation or oxidative stress.^{2,3} Symptoms of DPN in patients are heterogeneous, with approximately one-fourth of patients developing neuropathic pain, although the estimate varies between studies.^{4,5} The underlying mechanisms of DPN are not fully understood, but inflammation is believed to play a significant role.⁶ Unresolved inflammation has been linked to the development of DPN and neuropathic pain.^{7,8} Studies have also shown that macrophages play significant roles in the development of neuropathic pain in DPN and in the immune response associated with diabetes.^{9,10}

Ionized calcium-binding adaptor molecule 1 (IBA1) is a polypeptide particularly expressed in the cytoplasm of macrophages or microglia.^{11,12} The *IBA1* gene contributes in encoding the major histocompatibility complex (MHC) III, which is known to initiate inflammatory response in injury and in disease.^{11,13} Previous animal studies have shown that IBA1⁺ macrophages contribute to inflammatory responses in the central and peripheral nervous systems at early stages of diabetes.^{14,15} A previous study by our group examined the expression of IBA1⁺ macrophages in participants with type 2 diabetes and found an increase in IBA1⁺ macrophage infiltration in those with painful DPN, suggesting a link between IBA1⁺ macrophages and neuropathic pain in type 2 diabetes.¹⁶ However, the relationship between IBA1⁺ macrophages and neuropathic pain in type 1 diabetes is still unknown and warrants further investigation because there are several important differences between type 1 and type 2 diabetes.

Recent genetic studies suggest that Langerhans cells (LCs) are macrophages, regardless of their dendritic morphology.^{17,18} LCs reside in the outermost layer of skin, serving as the first line of defense for the body by detecting pathogens and changes in the environment and pass this information on to lymph nodes.¹⁹ Previous studies showed contradictory results in the relationship between LCs and cutaneous nerve fibers, with studies that showed close contact between LCs and nociceptors in epidermis,^{20,21}

others suggested no association between them.^{22,23} Clinical studies have revealed inconsistent effects of diabetes on LC expression. While some studies have shown a reduction in cutaneous LC density because of hyperglycemia, others have reported an increase in LC density in diabetic cornea and gingiva.²³⁻²⁵ Despite these findings, the relationship between LC expression and diabetes-induced neuropathic pain remains uncertain.

Our study aimed to investigate the relationship between cutaneous macrophages and neuropathic pain in DPN by visualizing and quantifying IBA1⁺ macrophages and LCs in skin biopsies from participants with type 1 diabetes, including those without DPN, with painless and painful DPN, and healthy controls. The goal was to gain a better understanding of the underlying mechanisms of DPN and neuropathic pain.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study was conducted in accordance with the principles outlined in the Helsinki Declaration II and received ethical approval from the Regional Ethics Committee (#1-10-72-103-19) and the Danish Data Protection Agency. Participants provided their informed consent for participation after having been fully informed about the study procedures.

Study Participants and Clinical Diagnosis of DPN and Pain

This study was part of a larger cross-sectional study that recruited 216 participants with type 1 diabetes and healthy controls from 3 Steno Diabetes Centers in Denmark from 2019 to 2021. We randomly selected 20 participants from each group (healthy controls, participants with type 1 diabetes but no DPN, and participants with type 1 diabetes and painless or painful DPN) and added additional participants to each group based on sex and age to ensure similarities between the groups. Inclusion criteria for participants with type 1 diabetes were a confirmed diagnosis of type 1 diabetes for at least 5 years and age 30–75 years. Participants were excluded from the study if they were unable to give informed consent, had neuropathy caused by known factors other than diabetes, experienced severe pain caused by factors other than

neuropathy, had a history of alcohol or substance abuse, were pregnant, had any skin disorder in the biopsy area, had prior chronic ulcers in the biopsy area, had intermittent claudication, had very weak/nonpalpable foot pulses, had existing diabetic foot ulcers, or had a known allergy to lidocaine. Inclusion criteria for the healthy controls was age 30–75 years, and exclusion criteria were equivalent to those for participants with type 1 diabetes, except that they could not have any known neurologic or metabolic disorders or be experiencing clinical depression or ongoing pain of any cause.

Neuropathy Assessment

All participants were thoroughly examined by using various methods, including ankle and knee reflexes, vibration detection threshold on the hallux using biothesiometer (where values over 25 V are considered abnormal), mechanical detection using Neuropen® (10 g monofilament) under the hallux (where 7 or fewer of 10 stimuli detected is considered decreased sensation), pinprick sensation using Neurotip® on the hallux, and temperature sensation using thermos rollers (Rolltemp II®) of 25°C (cold) and 40°C (warmth) on top of the hallux. In addition, participants were assessed using DPNCheck® that measures the amplitude and velocity of the sural nerve and is considered being a surrogate for a full nerve conduction study (amplitude <4 µV or velocity ≤40 m/s were considered abnormal result), 3 mm punch skin biopsy, and a panel of blood biomarkers, such as HbA1c, triglyceride, and total and LDL cholesterol. The diagnosis of DPN in the participants was performed by using the Toronto Diabetic Neuropathy Expert Group criteria, which include a combination of symptoms and signs of DPN.²⁶ Here, signs of DPN were assessed using the tests described earlier. The diagnosis was confirmed through abnormal IENFD results from skin biopsy and DPNCheck. Participants who reported pain in both feet/legs, reported sensory signs in the feet/legs, had a diagnosis of diabetes, and had abnormal IENFD or DPNCheck results were considered to have neuropathic pain. The level of pain was measured using a numeric rating scale (NRS) and the Neuropathic Pain Symptom Inventory questionnaire (NPSI). DPN severity was assessed using Toronto clinical neuropathy score (TCNS).

Skin Biopsy

Skin samples were obtained using a 3-mm punch biopsy technique (Miltex, York, PA) at the distal leg (10 cm above the lateral malleolus) and processed in accordance with established protocols.^{27,28} The samples were fixed overnight in Zamboni and 20% sucrose and cryoprotected overnight. They were then snap frozen and stored at –20°C before being cut into 50-µm thick sections for immunohistological staining.

Immunofluorescent Labeling and Imaging

All sections went through antigen retrieval in boiled sodium citrate buffer and incubated in water bath at 80°C for 20 minutes. The cooled sections were washed with washing buffer (0.3% Triton X-100 in 0.05M PBS buffer) and incubated with blocking buffer (1% bovine serum albumin in

washing buffer) for 1 hour. The primary antibody rabbit anti-IBA1 (FUJIFILM) with a concentration of 1:1,000 in blocking buffer was applied to skin sections overnight at 4°C, and secondary antibody donkey antirabbit-488 (1:800, Invitrogen) for 90 minutes at room temperature. The sections were rinsed with washing buffer and incubated with DAPI (1:5,000, Sigma) buffer, washed, and finally mounted on slide with 0.5% gelatine and fluorescent mounting medium and coverslipped.

Z-stack confocal images consisting of 15-image stacks with a 21-µm scanning thickness were acquired by a laser scanning spectral confocal microscope (Zeiss LSM 800, Germany) with a 40X oil lens. To reconstruct an image of the entire region of interest (ROI) in 1 skin section, the tile function was used to stitch all single-field images from the same skin section. A minimum of 2 individual skin sections from the same participant were imaged and used for IBA1 quantification.

Immunohistochemistry

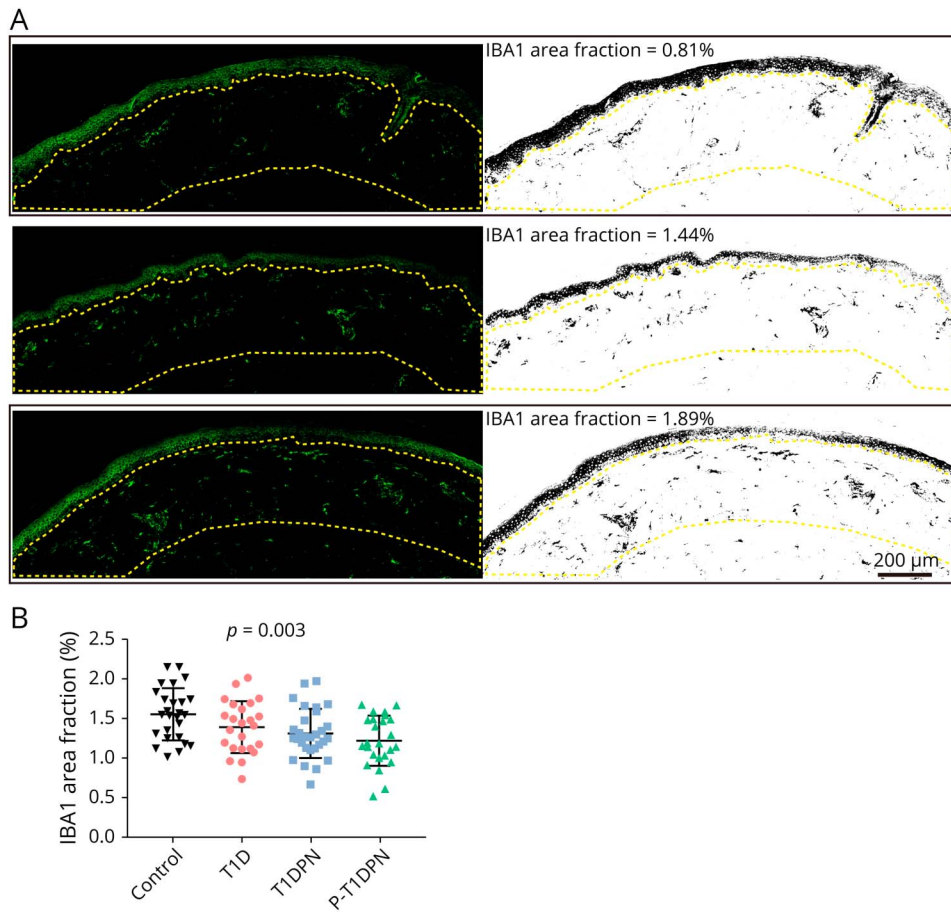
Antigen retrieval (Dako) and endogenous peroxidases blocking (3% H₂O₂) were performed before free-floating staining. After washing in washing buffer (0.3% Triton X-100 in 0.05M Tris-buffered saline), the sections were incubated with 1% bovine serum albumin in washing buffer for an hour, before overnight incubation of primary antibody rabbit anti-CD207 (1:500, Invitrogen). Sections were washed in washing buffer and then incubated with secondary antibody goat antirabbit-HRP (1:400, Dako) at room temperature for 2 hours. After rinsing sections with 0.3% TBST buffer, sections were treated with DAB working solution (0.05% DAK, 0.1% H₂O₂) for 5 minutes. Sections were rinsed in washing buffer and mounted on slides with gelatine. Skin sections were air dried on slides and rehydrated in distilled water and dehydrated in 70% ethanol (10 minutes), 90% ethanol (10 minutes), 99% ethanol (15 minutes), and xylene (15 minutes) and finally coverslipped.

Quantification

IBA1⁺ Macrophage Counting

IBA1⁺ macrophage quantification was analyzed on the original confocal Z-stack images by using the FIJI image-processing package based on ImageJ. 15 Z-stack images were orthogonally projected using Z projection function, and the average intensity of all stacks was shown in a 2-D image for the following measurement. Each image was adjusted by applying a specific intensity threshold to exclude the nonspecific labelling, so the remaining signal showed the positive labeling of IBA1⁺ macrophages. Then, the dermal IBA1⁺ macrophages were estimated by measuring the dermal IBA1 area fraction, which was counted by the area that was occupied by the total IBA1⁺ macrophages (IBA1_{area}), divided by the total region of interest (ROI) area (ROI_{area}), dermal area within 300 µm below epidermal-dermal border in the orthogonally projected 2-D confocal images. The IBA1 area fraction in each image was measured 3 times, and the average value of the 3

Figure 1 Immunofluorescent Labeling and Quantification of IBA1⁺ Macrophages in Human Skin Sections



(A) Representative images of different expression levels of IBA1⁺ macrophages in skin sections. (B) The quantification of IBA1 area fraction in dermis. Enclosed area by yellow dotted line: the region of interest (dermis that was 300 μm below epidermis-dermis border); T1D = type 1 diabetes without diabetic polyneuropathy; T1DPN = type 1 diabetes with painless diabetic polyneuropathy; P-T1DPN = type 1 diabetes with painful diabetic polyneuropathy.

individual double-blind measurements was used for the statistical analysis.

$$\text{IBA1 area fraction} = \frac{\sum a(\text{IBA1}_{\text{area}})}{\sum a(\text{ROI}_{\text{area}})}$$

Langerhans Cell Counting

Live counting was performed on Olympus BX50 light microscope (Olympus, Japan) with an automatic stage (Prior scientific), encoder (ND281, Germany), and digital camera (Olympus DP79, Japan) connected to a PC with the NewCAST software (ver. 4.4.5.0, Visiopharm A/S, Denmark).

1. LC number density was estimated by counting the total number of LCs in the epidermis, $Q^-(\text{LC})$, divided by the volume of epidermis, $\text{Epidermis}_{\text{volume}}$. The $\text{Epidermis}_{\text{volume}}$ was measured by multiplying the area of epidermis ($\text{Epidermis}_{\text{area}}$) and the section thickness (50 μm, disector height). The counting was performed using a 20X magnification lens.

$$N_V(\text{LC}) = \frac{\sum Q^-(\text{LC})}{\left(\sum a(\text{Epidermis}_{\text{area}}) * \text{Disector height}\right)}$$

2. LC process grading was estimated by a semiquantitative method with a novel grading system. The LC process levels were graded into 4 levels based on the number and length of the process in the skin sections. Two representative images for each process grading level are shown in Figure 2B. In detail, level 1 is LCs without process; level 2 is LCs with 1–2 processes; level 3 is LCs with 3–4 processes; and level 4 is LCs with more than 4 nicely spread processes. Process levels of 20 LCs were counted from each participant using a ×100 magnification lens.
3. LC soma cross-sectional area was presented by the maximum cross-sectional area of the LC soma, which was unbiasedly estimated using the 2-D Nucleator in the Visiopharm A/S software. For the counting of each soma, we first identified the largest cross-section of the soma in the skin and added 5 random 2-D isotropic test lines. We then marked at the cross between the test lines and the soma border. The length of the test lines within the soma were measured, which automatically generated the estimated cross-sectional area of the soma. The counting method is depicted in Figure 2C and described in previous studies.²⁹ Twenty LC somata were counted from each participant using a ×100 magnification lens.

Table 1 Patient Characteristics

Patient characteristics	Control (n = 26)	T1D (n = 24)	T1DPN (n = 29)	P-T1DPN (n = 25)	p Value
Female sex, n (%)	12 (46.2)	13 (54.2)	13 (44.8)	14 (56.0)	0.828
Diabetes duration, y (IQR)	—	26.1 (±14.0)	39.9 (±14.8)	34.3 (±11.7)	0.002 ^b
Age, y, (IQR)	58.6 (50.3–65.5)	50.4 (43.9–58.7)	63.3 (57.9–70.1)	60.2 (52.6–63.4)	0.005 ^b
BMI, kg/m ² , (IQR)	27.5 (24.6–35.6)	25.6 (24.0–28.2)	27.0 (23.3–30.8)	27.9 (23.8–31.2)	0.540
Smoking today, n (%)	0 (0.0)	1 (4.2)	0 (0.0)	3 (12.0)	0.089
Alcohol, 8 or more units/week, n (%)	5 (20.8)	5 (20.8)	8 (28.6)	4 (16.0)	0.757
Exercise, >1 times/week, n (%)	24 (100)	21 (87.5)	19 (67.9)	19 (76.0)	0.008 ^b
Antihypertensive, n (%)	2 (7.7)	10 (41.7)	22 (78.6)	15 (60.0)	0.000 ^c
Cholesterol-lowering medication, n (%)	1 (3.8)	14 (58.3)	27 (96.4)	20 (80.0)	0.000 ^c
IENFD, nerves/mm (IQR)	4.6 (2.8–5.6)	2.8 (1.7–4.2)	0.5 (0.0–1.7)	0.6 (0.0–2.3)	0.000 ^c
DPNCheck, abnormal, n (%)	5 (20.0)	3 (13.0)	25 (89.3)	21 (84.0)	0.000 ^c
DPNCheck, amplitude, μV	9.0 (6.0–12.0)	9.0 (6.0–13.0)	2.5 (0.0–4.0)	2.0 (2.0–4.0)	0.000 ^c
DPNCheck, conduction velocity, m/s	53.0 (50.0–56.0)	50.0 (44.0–54.0)	35.0 (0.0–45.0)	33.0 (25.0–42.0)	0.000 ^c
Neuropen, abnormal, n (%)	0 (0.0)	0 (0.0)	18 (62.1)	11 (44.0)	0.000 ^c
Neurotip, abnormal, n (%)	1 (3.8)	1 (4.2)	15 (51.7)	11 (44.0)	0.000 ^c
Warmth, abnormal, n (%)	0 (0.0)	1 (4.2)	17 (58.6)	12 (48.0)	0.000 ^c
Cold sensation, abnormal, n (%)	2 (7.7)	3 (12.5)	20 (69.0)	17 (68.0)	0.000 ^c
Biothesiometer >25 V, n (%)	0 (0.0)	2 (8.3)	15 (51.7)	15 (60.0)	0.000 ^c
Biothesiometry (V)	6.4 (5.6–9.9)	8.0 (4.8–14.3)	25.7 (17.8–41.7)	31.0 (17.3–50.0)	0.000 ^c
Pain, past 24 h (yes)	—	0.0 (0.0–0.0)	0.0 (0.0–0.0)	5.0 (5.0–6.0)	0.006 ^b
Pain, past 7 d (yes)	—	0.0 (0.0–0.0)	0.0 (0.0–0.0)	5.0 (5.0–7.0)	0.001 ^c
MNSI questionnaire score	2.0 (2.0–3.0)	2.5 (2.0–4.0)	5.0 (4.0–6.0)	7.0 (6.0–9.0)	0.000 ^c
MNSI clinical score	0.0 (0.0–1.0)	0.0 (0.0–1.0)	4.5 (3.0–6.0)	4.5 (3.0–6.0)	0.000 ^c
TCNS score	0.5 (0.0–1.0)	1.0 (0.0–2.0)	6.0 (4.0–8.0)	9.0 (7.0–10.0)	0.000 ^c
Systolic blood pressure, mm Hg (IQR)	136.2 (127.3–148.7)	136.8 (126.2–147.0)	131.2 (126.3–148.8)	140.0 (130.7–150.3)	0.562
Diastolic blood pressure, mm Hg, (IQR)	84.7 (77.7–93.0)	75.8 (73.0–82.5)	69.3 (65.8–81.2)	75.7 (72.3–84.3)	0.000 ^c
HbA1c, mmol/mol, (IQR)	35.6 (33.7–38.8)	55.5 (49.7–59.5)	62.0 (59.0–68.1)	64.0 (55.6–69.0)	0.000 ^c
Glucose mmol/L, (IQR)	5.5 (5.0–5.9)	8.6 (6.9–10.8)	8.0 (6.1–11.6)	9.3 (6.0–14.2)	0.000 ^c
Triglyceride, mmol/L, (IQR)	1.3 (1.0–1.7)	1.0 (0.8–1.3)	1.0 (0.7–1.5)	0.9 (0.7–1.2)	0.017 ^a
Total cholesterol mmol/L, (IQR)	5.6 (5.2–5.8)	4.3 (3.9–5.0)	4.5 (3.3–5.0)	4.3 (3.6–4.6)	0.000 ^c
LDL mmol/L, (IQR)	3.2 (±0.6)	2.3 (±0.7)	2.0 (±0.7)	2.0 (±0.6)	0.000 ^c
eGFR, mL/min/1.73 m ² , (IQR)	90.0 (86.8–90.0)	90.0 (90.0–90.0)	84.9 (72.7–90.0)	90.0 (86.5–96.0)	0.009 ^b
Urine albumin/creatinine ratio, mg/g, (IQR)	8.5 (4.4–15.7)	6.0 (3.8–8.0)	15.4 (8.1–42.0)	9.7 (5.0–19.7)	0.013 ^a

Abbreviations: BMI = body mass index; DPN = diabetic polyneuropathy; eGFR = estimated glomerular filtration rate; IENFD = intraepidermal nerve fiber density; LDL = low-density lipoprotein; MNSI = Michigan Neuropathy Screening Instrument; P-T1DPN = type 1 diabetes with painful diabetic polyneuropathy; T1D = type 1 diabetes without diabetic polyneuropathy; T1DPN = type 1 diabetes with painless diabetic polyneuropathy; TCNS = Toronto clinical neuropathy score.

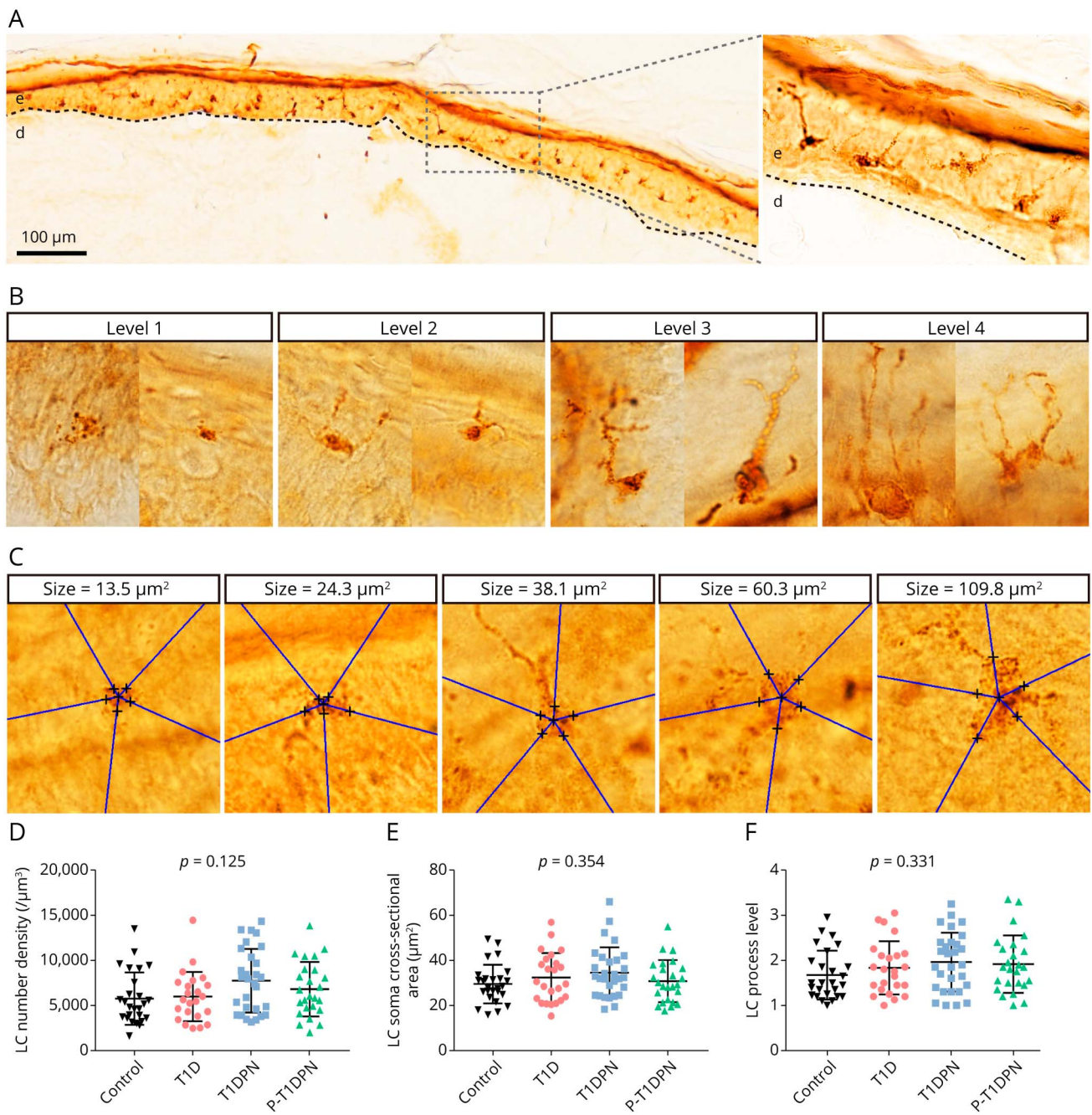
Categorical variables are presented as the frequency and the percentage of x within the group, x (%). Normally distributed continuous variables are presented as mean (±SD). Non-normally distributed continuous variables are presented as median value and interquartile range (IQR). For categorical variables, the Fisher exact test was conducted. For continuous variables, 1-way ANOVA (normally distributed variables) and Kruskal-Wallis H test (non-normally distributed variables) were conducted.

^a p values <0.05.

^b p values <0.01.

^c p values <0.001.

Figure 2 Quantification of Cutaneous Langerhans Cell Morphologies



(A) Representative image of Langerhans cells in healthy human epidermis. (B) Representative structures of 4 Langerhans cell process levels. (C) Representative images of Langerhans cell soma cross-sectional area estimation. (D) The quantification of Langerhans cell number density in epidermis. (E) The quantification of Langerhans cell soma cross-sectional area in epidermis. (F) The quantification of Langerhans cell process levels in epidermis. LC = Langerhans cell; T1D = type 1 diabetes without diabetic polyneuropathy; T1DPN = type 1 diabetes with painless diabetic polyneuropathy; P-T1DPN = type 1 diabetes with painful diabetic polyneuropathy.

Statistical Analysis

The distribution of all variables was checked using histograms and quantile-quantile plots. Data were presented as mean (\pm SD) for normally distributed variables and medians (interquartile range, IQR) for non-normally distributed variables. Comparison between all groups was performed using parametric test (1-way ANOVA) or nonparametric test (Kruskal-Wallis H), depending on the data distribution.

p values were presented as raw values and adjusted values for post hoc analysis. Adjustments for sex, age, and HbA1c were performed using a multiple linear regression model. All non-normal variables were transformed to obtain a normal distribution, using either \log_{10} or square root transformations. Correlation analysis was performed using the Pearson correlation (in case some data were not normally distributed, they were transformed to achieve normal distribution). Partial

Table 2 Cutaneous Immune Cell Quantifications and Comparisons in Study Groups

Immune cell quantification	Control (n = 26)	T1D (n = 24)	T1DPN (n = 29)	P-T1DPN (n = 25)	p Value
IBA1 area fraction, %, (\pm SD)	1.6 (\pm 0.3)	1.4 (\pm 0.3)	1.3 (\pm 0.3)	1.2 (\pm 0.3)	0.003 ^a
LC number density, mm ⁻³ , (IQR)	4,987.3 (3,602.8–7,544.1)	5,827.4 (4,063.2–7,395.4)	7,938.1 (4,600.3–10,338.2)	6,037.9 (4,745.9–8,924.1)	0.125
LC cross-sectional soma area, mm ⁻² , (IQR)	28.2 (24.8–31.8)	30.0 (22.6–39.6)	32.3 (24.5–40.0)	29.3 (22.6–36.5)	0.354
LC process level, (IQR)	1.5 (1.2–2.0)	1.8 (1.4–2.1)	1.9 (1.4–2.4)	1.9 (1.5–2.3)	0.331

Abbreviations: LC = Langerhans cell; P-T1DPN = type 1 diabetes with painful diabetic polyneuropathy; T1D = type 1 diabetes without diabetic polyneuropathy; T1DPN = type 1 diabetes with painless diabetic polyneuropathy. Normally distributed continuous variables are presented as mean (\pm SD). Non-normally distributed continuous variables are presented as median values and interquartile ranges (IQR). Statistical differences were tested, using 1-way ANOVA (normally distributed variables) and the Kruskal-Wallis H test (non-normally distributed variables).
^a *p* values <0.01.

correlations were performed to adjust for confounding variables including age, sex, and HbA1c. Statistical significance (α) was set at a *p* value \leq 0.05.

Data Availability

Deidentified data are available from the corresponding author on request pending signed agreements from Aarhus University.

Results

Participant Characterization

A total of 78 participants (24 participants without DPN, 29 with painless DPN, and 25 with painful DPN) and 26 healthy controls were included in the study. Table 1 summarizes the main clinical characteristics of the participants. There were statistically significant differences in several of the baseline characteristics (Table 1), including age, diabetes duration, HbA1c, total cholesterol, LDL, eGFR, diastolic blood pressure, and urine albumin/creatinine ratio. As for pathophysiologic tests of DPN, there were differences in IENFD and DPNCheck between the groups (all *p* < 0.001). Among patients with DPN, more than 80% had abnormal DPNCheck results and more than 90% had abnormal IENFD (data not shown), indicating that most patients had both large and small fiber abnormalities. Bedside tests such as mechanical, vibration, and thermal sensation measured by pinprick, biothesiometer, and thermal rollers differed between the groups, as did the MNSI questionnaire (MNSI-Q) and MNSI total score and TCNS (all *p* < 0.001). Last, the median pain intensity (NRS) was 5 (5;6) and 5 (5;7) in P-T1DPN at 24 hours and 7 days, respectively, before performing the sensory test and skin biopsy acquisition.

Visualization and Quantification of Cutaneous IBA1⁺ Macrophages

The presence of dermal IBA1⁺ macrophages was visualized by immunofluorescent labeling. The IBA1 area fraction was measured within 300 μ m of the dermal-epidermal border, and results were compared between groups. Representative images of low and high dermal IBA1⁺ macrophage expression

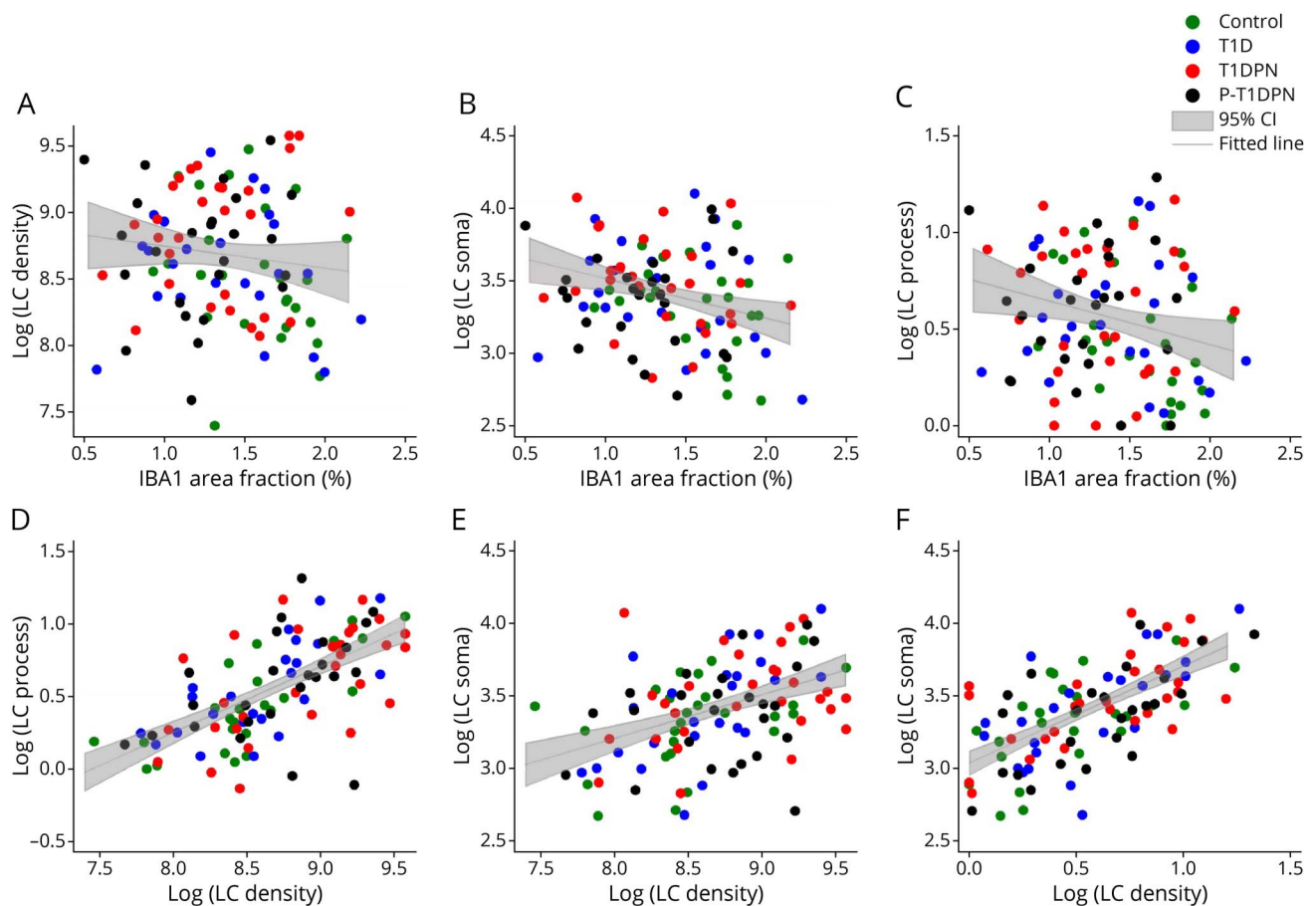
are shown in Figure 1A. The results showed a statistically significant difference in IBA1 area fraction (%) between groups (*p* = 0.003), with healthy controls having the highest expression (1.6 \pm 0.3%), followed by participants without DPN (1.4 \pm 0.3%), patients with painless DPN (1.3 \pm 0.3%), and last, patients with painful DPN (1.2 \pm 0.3%) (Figure 1B, Table 2). Post hoc analysis showed a decrease in IBA1 area fraction in T1DPN and P-T1DPN groups compared with healthy controls before adjusting for confounders (both *p* < 0.05; eTable 1, links.lww.com/NXI/A881), but no difference was observed between the other 2 groups (all *p* > 0.05; eTable 1). After adjusting for confounders (age, sex, and HbA1c), a reduction of IBA1 was observed in P-T1DPN compared with healthy controls (*p* = 0.050; eTable 1).

Visualization and Quantification of Cutaneous Langerhans Cells

LCs were visualized in epidermis by immunohistochemistry labeling of langerin (CD207⁺). Representative images of different LC morphologies are shown in eFigure 1 (links.lww.com/NXI/A881). The expressional differences of LCs vary not only in number density but also in soma and processes. Therefore, the LC cell number density, soma cross-sectional area, and process level were analyzed separately for better evaluation of LC characteristics (Figure 2). There was no statistically significant difference between all groups when analyzing LC cross-sectional soma area and process level with ANOVA (both *p* > 0.05; Table 2). However, there was a weak but not statistically significant trend (*p* = 0.125) toward a difference in LC density between the groups, with higher LC number density seen in participants with diabetes (Table 2).

Post hoc analysis showed no significant difference in the 3 LC quantifications between any 2 groups before adjusting for confounders (all *p* > 0.05; eTables 2–4, links.lww.com/NXI/A881). After adjusting for confounders (age, sex, and HbA1c), significant differences were observed only in LC number density: (1) between those with T1DPN and healthy controls or those with type 1 diabetes without DPN (both *p* < 0.05); (2) between those with P-T1DPN and healthy controls

Figure 3 Correlations of Immune Cell Quantifications in all Participants



(A–C) Correlations between IBA1 area fraction and LC number density (A), LC soma cross-sectional area (B), and LC process level (C). (D–E) Correlations between 3 LC quantifications: (D) correlation between LC number density and LC process level (E) correlation between LC number density and LC soma cross-sectional area; and (F) correlation between LC process level and LC soma cross-sectional area. LC_density = LC number density; LC_soma = Langerhans cell soma cross-sectional area; LC_process = Langerhans cell process level.

($p = 0.019$); and (3) no difference was observed between other groups (all $p > 0.05$) (eTable 2).

Correlations Between Experimental Quantifications

Weak and negative correlations were found between IBA1 area fraction and LC soma cross-sectional area and LC process level (all $p < 0.05$), but not LC number density ($p = 0.237$; Figure 3, A–C, eTable 5, links.lww.com/NXI/A881). Furthermore, the 3 LC morphological quantifications, LC number density, LC soma cross-sectional area, and LC process level, were correlated with each other, meaning that if one LC quantification was high, the two others were high as well, and vice versa. (all $p < 0.001$; Figure 3, D–F, eTable 5).

Correlations Between Experimental Quantifications and Measures of Neuropathy

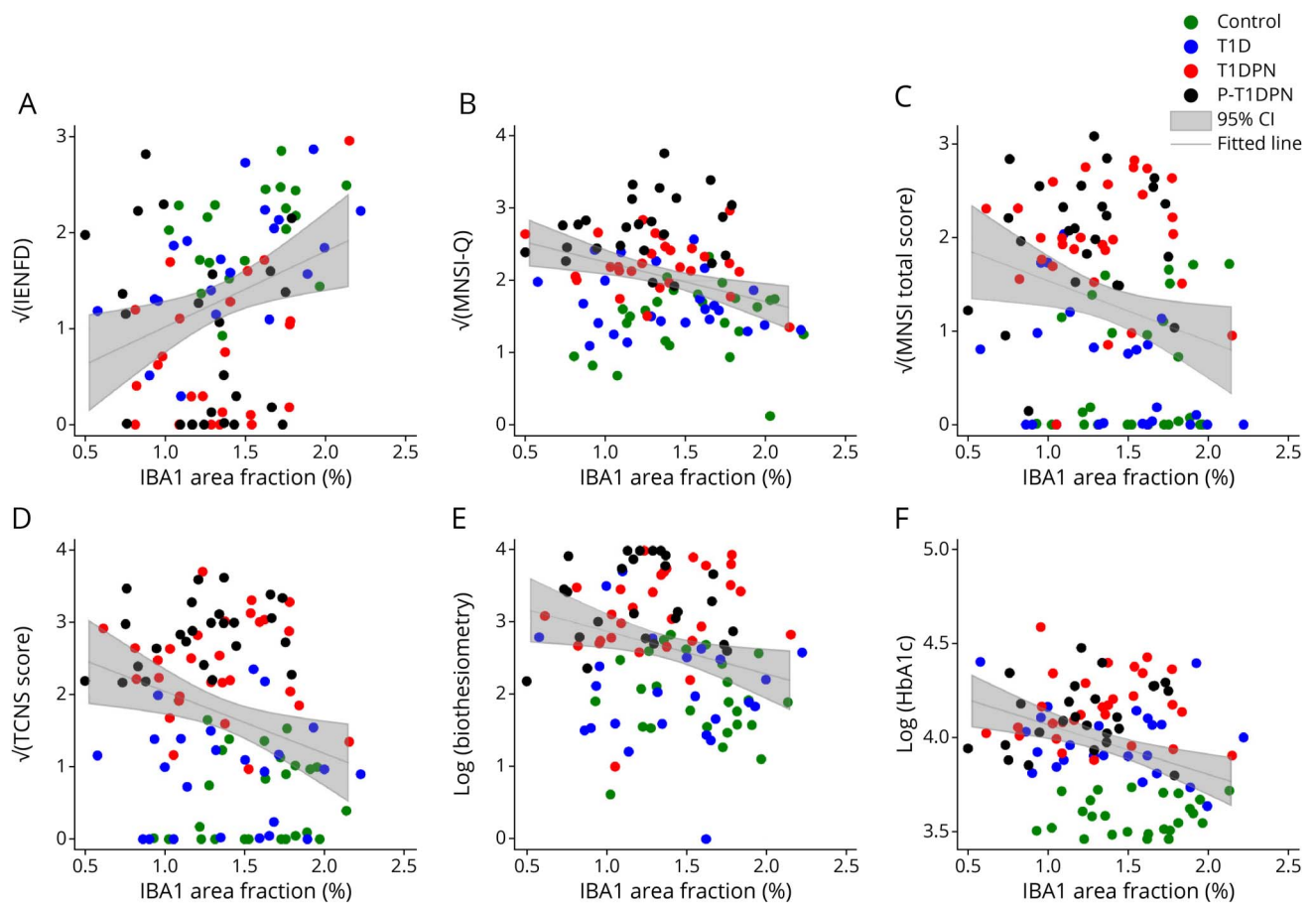
We examined the correlation between LCs and IBA1⁺ macrophages with measures of DPN. There was a positive correlation between IBA1 area fraction and IENFD ($r = 0.29$, $p = 0.007$) and weak but significant negative correlations between

IBA1 area fraction and MNSI-Q ($r = -0.29$, $p = 0.003$), MNSI-total ($r = -0.22$, $p = 0.023$), TCNS ($r = -0.26$, $p = 0.009$), and biothesiometer threshold ($r = -0.23$, $p = 0.017$) in the whole study cohort (Figure 4, A–E, eTable 5, links.lww.com/NXI/A881). IBA1 area fraction also correlated with HbA1c levels ($r = -0.32$, $p = 0.001$, Figure 4F), and only the correlation between IBA1 area fraction and IENFD remained statistically significant after adjusting for age, sex, and HbA1c ($r = 0.23$, $p = 0.037$, eTable 6). No correlation was found between LC morphology and the abovementioned measures of DPN (eFigure 2, eTables 5–6).

Discussion

In this study, we visualized and quantified 2 cutaneous immune cells, epidermal LCs and dermal IBA1⁺ macrophages, in carefully phenotyped participants with type 1 diabetes, without and with DPN, the latter with and without pain, and healthy controls without diabetes. We observed significant differences in IBA1⁺ macrophage infiltration between the

Figure 4 Correlations Between IBA1 Area Fraction and Neuropathy Measurements



(A) Correlation between IBA1 area fraction and intraepidermal nerve fiber density (IENFD). (B) Correlation between IBA1 area fraction and Michigan Neuropathy Screening Instrument Questionnaire (MNSI-Q). (C) Correlation between IBA1 area fraction and Michigan Neuropathy Screening Instrument total score (MNSI total score). (D) Correlation between IBA1 area fraction and Toronto Clinical Neuropathy score (TCNS score). (E) Correlation between IBA1 area fraction and vibration test (Biothesiometer). (F) Correlation between IBA1 area fraction and HbA1c.

groups, with a decrease in IBA1⁺ macrophage infiltration in patients with DPN. The difference, while not large in absolute values, remained statistically significant after adjusting for confounders (age, sex, and HbA1c). Furthermore, IBA1⁺ macrophages showed a statistically significant positive correlation with IENFD and 2 of 3 LC measurements (LC somata and processes) but a weak negative correlation with MNSI-Q, MNSI total score, neuropathy severity (TCNS score), and vibration detection. However, after adjusting for confounders, only a weak positive association between IBA1⁺ macrophages and IENFD remained statistically significant. We found no differences in the density or morphology of LCs between the groups. Taken together, while we found associations between IENFD, macrophages, and LCs, the skin of patients with longstanding type 1 diabetes and DPN is not increasingly innervated by these immune cells.

These findings differ from our recent findings in patients with relatively newly diagnosed (5 to 6 years) screen-detected type 2 diabetes and DPN, where we demonstrated higher IBA1⁺ macrophage density in patients with painful DPN compared

with those without pain and healthy controls and where we found an increasing gradient of IBA1⁺ macrophages, with healthy controls without diabetes having the lowest density, followed by patients with painless DPN, and last, patients with painful DPN.¹⁶ The patient cohorts were in many ways similar, because HbA1c levels, hypertension treatment, pain intensity, and TCNS scores were comparable between the 2 cohorts. There were minor disparities in age, BMI, and smoking status. The differences between these 2 findings could potentially be explained by different types of diabetes and a vast difference in diabetes duration (with a median value up to 40 years in this study compared with up to 6 years in the type 2 diabetes study). Furthermore, while inflammation plays a role in diabetic neuropathy, there are important pathophysiologic differences between type 1 and type 2 diabetes.³⁰ In type 1 diabetes, the immune system attacks and destroys the insulin-producing beta cells in the pancreas, leading to a loss of insulin production.^{31,32} This chronic lack of insulin can lead to high blood glucose levels, which can damage the nerve fibers, leading to diabetic neuropathy.³³ In type 2 diabetes, on the other hand, the cells do not respond

properly to insulin, leading to high blood glucose levels.³⁴ However, besides hyperglycemia, other metabolic dysfunctions including hyperlipidemia, hypertension, and obesity are more often seen in type 2 diabetes.^{35,36} Those multiple metabolic dysfunctions in type 2 diabetes may lead to chronic low-grade inflammation, which is believed to play a role in the development of diabetic neuropathy.^{37,38} Furthermore, inflammation in the blood vessels can lead to a decrease in blood flow, which further leads to nerve damage.^{39,40} Taken together, while inflammation may also contribute to the development of diabetic neuropathy in type 1 diabetes, through an increase in the production of cytokines, this does not seem to be reflected by changes in IBA1⁺ macrophages or LCs in the skin of patients with DPN accompanying type 1 diabetes, at least not in patients with longstanding diabetes.

This study builds on our previous research in patients with type 2 diabetes by including an analysis of the main epidermis-resident immune cell, the Langerhans cells. LCs are found in the epidermis and mucosal membranes and play a crucial role in the immune system as the "health guardians" of peripheral tissue.^{18,19} However, with previous studies mainly focusing on the density changes of LCs, the relationship between LC morphological changes and pathologic changes is not well understood. Our results revealed that the morphology of LCs, including cell number density, processes, and soma cross-sectional area, varied among the study participants. Correlational analysis showed a positive relationship between the 3 LC morphological characteristics, suggesting degeneration of LCs affects the entire cell and its processes, as represented by number density, soma size, and process density. But no correlation was found between LC morphology and any clinical neuropathy markers in the entire study cohort. In addition, there were no statistically significant differences in LC morphology between the study groups, indicating that there is no clear relationship between LC expression and DPN or neuropathic pain.

Clinical studies have also reported conflicting results in LC expression in diabetes, and this is not the first study reporting a decrease in the skin of patients with diabetes. One study found a significant decrease in epidermal LC density in adult patients with early-diagnosed type 2 diabetes compared with controls, with no correlation between LC density and IENFD or clinical signs.²³ A second study assessed LC density using corneal confocal microscopy in type 1 and type 2 diabetes and found that compared with healthy controls without diabetes, patients with early or mild neuropathy were more likely to have increased LCs in the cornea while patients with progressed neuropathy showed a reduction in LC density compared with healthy controls and those with early or mild neuropathy.⁴¹ A third study, however, found an increase in epidermal LC density in adult patients with neuropathic pain induced by type 1 (n = 1) or type 2 (n = 12) diabetes compared with healthy controls, but no correlation between LC number density and pain intensity was found.⁴² It is worth

noting that patients in this previous study had a long diabetes duration with a mean value of 14 years,⁴² but the diabetes duration in this study is twice as long and includes only patients with type 1 diabetes. These contradicting results may be due to differences in study design, study cohorts, and type and duration of diabetes. The complexity of the disease and the different stages of the diabetes may also contribute to the inconsistent findings.

The findings in the literature suggest that LCs may primarily play a role in the early phase of neuropathy and may be supported by preclinical studies. Animal studies have shown both a decrease and an increase in cutaneous LC density in mice with type 1 and type 2 diabetes, respectively. One study reported a decrease in LC density in type 1 diabetes mice at 16 weeks postinduction compared with control mice, while another study reported both a decrease and an increase in LC density in type 2 diabetes mice at different stages.^{20,43} It is important to note that animal models of type 1 diabetes are typically induced using streptozotocin which are toxic to the beta cells, which does not resemble the human autoimmune condition.⁴⁴

The potential relationship between lower concentrations of IBA1⁺ macrophages in the skin and the development and progression of diabetic neuropathy in type 1 diabetes is worth considering. These immune cells are critical in regulating inflammation and oxidative stress in peripheral nerves, and they also play a role in removing cellular debris and waste products that can accumulate and contribute to nerve damage over time.^{45,46} While the exact mechanisms linking lower concentrations of IBA1⁺ macrophages to diabetic neuropathy are not yet fully understood and require further research, these data suggest that a reduction in these immune cells could be a contributing factor in the development and progression of autoimmune-induced diabetic neuropathy.

Patients with DPN in this study had relatively mild DPN (with median TCNS of 6 for those without pain and 9 for those with pain) and those with pain had relatively mild to moderate pain (a median of 5 over the last 7 days), resulting in that most patients did not experience severe DPN. Inclusion of more patients with more severe symptoms of DPN may have resulted in more pronounced differences between the groups. In addition, during the random selection of 24–29 patients from each group, while we tried to consider sex and age of the participants, we were unable to completely match the groups of patients. However, the influence these confounders might have had on the results were reduced by adjusting for them. Even so, there were differences between the groups in several patient characteristics that may have influenced the results that we were unable to adjust for. Limitations in the skin biopsy analysis include that we did not measure other parameters related to immune cells, such as the activation status of macrophages or the levels of blood and tissue-resident cytokines. Last, immunohistological and stereological methods can assess only structural changes, and

potential functional alterations of the targeted immune cells cannot be addressed by this study. Strengths of the study include carefully phenotyped patients following recommended guidelines and the well-developed immunohistochemical staining and stereological quantification methods.

Glutamic acid decarboxylase (GAD) enzymes are frequently detected in type 1 diabetes, serving as markers for the autoimmune destruction of pancreatic beta cells.⁴⁷ Furthermore, they are strongly associated with stiff person syndrome, a condition characterized by painful spasms that affect approximately 30% of patients with type 1 diabetes.^{47,48} Considering the high prevalence of painful spasms in type 1 diabetes and the findings of this study suggesting a lesser role of the innate immune system compared with that in type 2 diabetes, which is primarily driven by the adaptive immune system, it would be worthwhile for future investigations to explore the potential relationship between macrophages, GAD antibodies, and painful spasms in type 1 diabetes to further understand possible mechanism-based treatment options.⁴⁹

Moreover, future studies should include a larger cohort of patients to allow both comparison of patients with similar characteristics and comparison of patients with different degree of neuropathy. In addition, future studies should assess immune cells in other etiologies, e.g., chemotherapy-induced neuropathy, and additional biomarkers (including systemic markers such as serum cytokines) to further investigate the role of immune cells in neuropathic pain.

The main finding of this study is that the IBA1⁺ macrophage expression in peripheral skin biopsies is reduced in this cohort of patients with type 1 diabetes with long-term diabetes diagnosis compared with that in controls and that this reduction is more pronounced in patients with type 1 diabetes with diabetic neuropathy. The reduction in IBA1 area fraction is furthermore correlated with several different measures of neuropathy. These data suggest that reduction in these immune cells may play an important role in the development and progression of autoimmune-induced diabetic neuropathy.

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Continued

Appendix (continued)

Name	Location	Contribution
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