Pronounced reduction of cutaneous Langerhans cell density in recently diagnosed type 2 diabetes

*Short title:* Loss of epidermal Langerhans cells in diabetes

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ABSTRACT

Immune-mediated processes have been implicated in the pathogenesis of diabetic polyneuropathy. Langerhans cells (LCs) are the sole dendritic cell type located in the healthy epidermis and exert tolerogenic immune functions. We aimed to determine whether alterations in cutaneous LC density and intraepidermal nerve fiber density (IENFD) are present in patients with recently diagnosed type 2 diabetes. Skin biopsies from the distal leg from 96 type 2 diabetic patients and 75 healthy controls were used for quantification of LC density and IENFD. LCs and IENFs were labeled using immunohistochemistry. Nerve conduction studies, quantitative sensory testing, and neurological examination were used to assess peripheral nerve function. LC density was markedly reduced in the diabetes compared to the control group, but did not correlate with reduced IENFD or peripheral nerve function. Multivariate linear regression analysis revealed a strong association between LC density and whole-body insulin sensitivity in women but not men with diabetes. Prospective studies should establish whether the pronounced reduction of cutaneous Langerhans cells detected in recently diagnosed type 2 diabetes could promote a cutaneous immunogenic imbalance towards inflammation predisposing to polyneuropathy and foot ulcers.
Recent evidence suggests the involvement of inflammatory processes in the pathogenesis of type 2 diabetes (T2D) (1) and its complications such as polyneuropathy (2). Diabetic neuropathy is associated with extensive morbidity, increased mortality and reduced quality of life (3). Distal leg skin biopsy with quantification of intraepidermal nerve fiber density (IENFD) is a reliable and efficient technique to assess the diagnosis of small fiber neuropathy (SFN) (4). However, the pathophysiological processes triggering the loss of IENFs in diabetic neuropathy are unknown. Langerhans cells (LCs) are the sole dendritic cells located in the healthy epidermis. It has been shown that IENFs have an immunemodulatory impact on LCs (5). This finding indicates a close relationship between the nervous system and the function of distinct immune system components. In patients with type 1 diabetes, cutaneous LC density was markedly reduced immediately at diabetes onset, but not in those with a known diabetes duration of 6 months (6). An increase in LC density has been reported in diabetes patients with painful SFN, but not in glucose tolerant persons with painful or painless SFN (7) or those with neuropathic pain due to postherpetic neuralgia (8). However, these studies used rather small samples of subjects. It is unknown whether LC density is altered in relation to IENFD early during the course of T2D. Early detection of putative neuro-immune alterations could be useful in developing strategies to prevent clinically advanced neuropathy and foot ulcers. Therefore, we aimed to assess LC density and IENFD and their relationship to metabolic factors in patients with recently diagnosed T2D.

**RESEARCH DESIGN AND METHODS**

**Subjects.** The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Heinrich Heine University, Düsseldorf. All participants provided a written informed consent. Healthy control subjects (n=75) and patients with recently diagnosed T2D (n=96) were studied. Subjects with T2D were participants of the
prospective German Diabetes Study (GDS) which evaluates the long-term course of diabetes and its sequelae (9). Inclusion criteria for entry into the GDS were type 1 or type 2 diabetes, known diabetes duration ≤1 year, and age 18-69 years. Exclusion criteria were secondary diabetes, pregnancy, severe diseases (cancer), psychiatric disorders, immunosuppressive therapy, limited cooperation ability, and neuropathy from causes other than diabetes. Inclusion criteria for the control group were age ≥18 years, while exclusion criteria corresponded to those applied to the diabetic group, except for an abnormal oral glucose tolerance test (10) and neuropathy from any cause.

**Hyperinsulinemic euglycemic clamp**

The hyperinsulinemic euglycemic clamp was performed in diabetes patients according to the Botnia protocol as previously described (11). In brief, following a 60 min intravenous glucose tolerance test, study participants received a 10-min insulin bolus (40 U/h) and continuous insulin infusion (40 mU×m²×min⁻¹). Plasma glucose was adjusted with 20% glucose enriched with 2% D-[6,6-²H₂]glucose to 5.0 mmol/L. Whole body glucose disposal (insulin sensitivity) was calculated as M-value as previously reported (12).

**Peripheral nerve function**

Peripheral nerve function tests were performed as previously described (13). Motor nerve conduction velocity (MNCV) was measured in the median and peroneal nerves, while sensory nerve conduction velocity (SNCV) was determined in the median and sural nerves at a skin temperature of 33-34°C using surface electrodes (Nicolet VikingQuest, Natus Medical, San Carlos, CA). Quantitative sensory testing was evaluated by vibration perception threshold (VPT) at the second metacarpal bone and medial malleolus using the method of limits
(Vibrameter, Somedic, Stockholm, Sweden) and by thermal detection thresholds (TDT) including warm and cold thresholds at the thenar eminence and dorsum of the foot using the method of limits (TSA-II NeuroSensory Analyzer, Medoc, Ramat Yishai, Israel). Neurological examination was performed using the Neuropathy Disability Score (NDS) and Neuropathy Symptom Score (NSS) (14).

**Skin biopsy and tissue fixation.** Three millimeter skin punch biopsies were taken under local anesthesia from the left lateral calf, approximately 10cm proximal to the lateral malleolus. The tissue was fixed with 2% periodate-lysine-paraformaldehyde (PLP) at 4 °C for 24h, rinsed 2×10min with 0.1M Sorensen buffer and incubated in 33% sucrose for 3h. After cryoprotection with 0.02M Sorensen buffer containing 20% glycerol at 4 °C overnight tissue was stored at -80 °C (4,15).

**Immunohistochemistry.** Serial sections of skin biopsies at 50 µm thickness for IENF detection and at 10 µm for LC detection were cut perpendicular to the skin. Staining of IENF was performed following the free floating method as described before (15) with some modifications. In brief, after blocking sections were incubated with a rabbit anti-PGP9.5 antibody (Millipore, Temecula, CA) and a biotinylated anti-rabbit IgG antibody (Vector Labs, Burlingame, CA) for 1h, followed by 1h incubation with Vector ABC kit and 3min with Vector SG substrate kit. All steps were performed at room temperature. For the detection of LCs, sections were blocked, incubated with the mouse anti-langerin [12D6] antibody (Abcam, Cambridge, U.K.) overnight and a biotinylated anti-mouse IgG antibody (Vector Labs, Burlingame, CA) for 45min, followed by incubation with Vector ABC and Vector SG substrate. All steps were performed at room temperature.
**Quantification of IENFD and LC density.** For the quantification of IENFD, a method adopted by the European Federation of Neurological Sciences was used (16). Individual IENFs from 4 cross sections per subject were visually counted along the length of the epidermis using a Leica DMRBE inverted microscope (Leica, Wetzlar, Germany) equipped with an Olympus DP73 digital color camera (Olympus, Hamburg, Germany), and cellSens imaging software v1.7 (Olympus Europa, Hamburg, Germany). Only the IENFs crossing the dermal-epidermal border were counted.

For the quantification of LC density, two sections per subject were used and the mean was calculated. LCs in the epidermis were visually counted using the 40x objective and cellSens software.

**Statistical analyses.** For normally distributed data parametric tests (t-test or Pearson product-moment correlation), otherwise nonparametric tests (Mann-Whitney U test or Spearman rank correlation) were applied. To determine possible correlations between two variables multiple linear regression analyses were performed. Continuous data were expressed as median and interquartile range or mean±SD. Categorical data are given as percentages of subjects. The level of significance was set at α=0.05.

**RESULTS**

Anthropometric, demographic and neurological measures in the groups studied are shown in Table 1. Diabetes patients had a higher BMI than control persons. Out of 96 patients, 28 (29.2%) were overweight and 60 (62.5%) were obese. Peroneal MNCV, sural SNCV, and cold TDT were lower, while malleolar VPT and NSS were higher in the diabetes than in the control group. In the diabetes patients, HbA1c was 6.3 (1.1)% or 45.4 (7.8) mmol/mol, M-
value was 5.04 (3.05), and known duration of diabetes until skin biopsy was 12.0 (12.0) months. The percentages of patients receiving diet only, oral glucose-lowering drugs, and insulin were 44.8, 51.0, and 4.2%, respectively.

IENFD was lower in the diabetes than in the control group (7.66 (4.06) vs. 9.22 (4.32) fibers/mm; \( P=0.002 \)) (Figure 1A). The analyses of the LC density (Figure 1B) revealed a pronounced LCs reduction in diabetes patients compared to controls (387 (220) vs. 563 (273) cells/mm\(^2\); \( P<0.0001 \)). The difference in LC density between the groups remained significant after adjustment for age, sex, BMI and smoking (\( \beta=-0.313; P<0.0001 \)).

Correlation analyses did not reveal a group or gender specific relationship between IENFD and LC density. However, a sex specific correlation of LC density with M-value as a measure of insulin sensitivity was observed in diabetic women (\( R^2=0.435; P<0.0001 \)) (Figure 2A). The correlation remained strongly positive after adjustment for age, BMI, smoking, known diabetes duration, and HbA1c (\( \beta=0.749; P=0.001 \)) (Table 2). In contrast, there was no correlation of LC density with M-value in diabetic men (Figure 2B). There was no difference between diabetic men and women in M-value [5.09 (2.95) vs. 5.04 (4.07) mg/kg\(^*\)min] and HbA1c [6.3 (1.1) vs 6.3 (1.2)% or 45.4 (7.6) vs 45.4 (8.6) mmol/mol].

**DISCUSSION**

There is evidence to suggest that chronic subclinical inflammation is implicated in the pathogenesis of T2D (1,17,18). We previously demonstrated in a population-based study that subclinical inflammation is also associated with diabetic polyneuropathy (2). However, it is unknown whether components of the immune system are related to IENF loss in diabetic SFN. Epidermal LCs are the sole dendritic cells of a healthy epidermis and their function is in close relationship with IENFs (5). Recently, it became evident that LCs, which essentially
represent a subset of dendritic cells, have a unique feature of maintaining immune tolerance (19,20). We describe here a striking reduction of LC density in patients with recently diagnosed T2D. Because LCs exhibit anti-inflammatory properties, the reduction of epidermal LC density should result in a shift to a pro-inflammatory cutaneous environment. If inflammation is involved in diabetic SFN, one would expect a direct relationship between LC density and IENFD. However, we did not find such an association in this population. This suggests that, at least in recently diagnosed T2D, reduced LC density may not affect IENFD. This notion is in accordance with a recent experimental study reporting that the effect of diabetes on LC proliferation and maturation in rats was independent of effects on cutaneous innervation (22). However, it is possible that there is a time dependent impact of reduced LC density on small nerve fibers, which can only be detected in a long-term prospective study. On another note, diabetes and diabetic polyneuropathy predispose to foot infections and ulcers. It is tempting to speculate that foot infections and ulcers could be predicted by the distinct loss of LCs observed herein.

The results of the present study differ from those of a recent study reporting an increase in LC density in a small group of diabetes patients with painful SFN (7). Moreover, in that study, the presence of diabetes was associated with increased LC density, and IENFD was inversely related to LC density. In contrast, we demonstrate that recently diagnosed T2D is characterized by a marked reduction of LCs in the lower limbs which is not related to IENFD. Interestingly, the mean LC number in the subjects with diabetes studied by Casanova-Molla et al. (7) was similar to the LC count in the present study. However, in their control subjects LC density was considerably lower than in ours. We have no explanation for this discrepancy, but the LC number established in our control subjects corresponds to the findings of several previous studies (6,21,22).
An unexpected finding was the strong positive correlation between the M-value as a measure of insulin sensitivity and LC density in diabetic women. It has been shown previously that inflammatory markers were stronger predictors of incident T2D in women than in men (23). One of the reasons for this finding could be the sexual dimorphism in the immune response in humans, e.g. females produce more vigorous cellular and humoral immune reactions and are more resistant to certain infections (24). LCs maintain immune tolerance in normal skin by selectively and specifically inducing activation and proliferation of skin resident regulatory and memory T cells (19,20). Inflammation is known to associate with insulin resistance (25). The reduction of LC density could result in an impairment of skin tolerance maintenance and therefore to a cutaneous pro-inflammatory environment shift. This could be one possible explanation for the correlation of LC density with insulin sensitivity in diabetic women.

The strength of the present study is the large number of subjects available for morphometric analyses. However, the study has also two limitations. First, because M-value was not available in the control group, we could not determine whether insulin sensitivity associates with LC density also in glucose tolerant women. Second, the cross-sectional nature of the present analysis does not allow to assess the predictive value of the described reduced LC density and IENFD. However, prospective analyses in the present diabetic and control groups will be performed at the 5-year and 10-year follow-up of the GDS.

In conclusion, we demonstrate that patients with recently diagnosed T2D show a striking reduction of LC density. In women, LC density is associated with insulin sensitivity. Because LCs promote cutaneous immune tolerance, long-term prospective studies will determine whether their reduction could be a predisposing factor for cutaneous infection and ulceration in diabetic patients.
ACKNOWLEDGMENTS

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No potential conflicts of interest relevant to this article were reported.

D.Z. and A.S designed the experiments. J.B., I.Z and K.J. conceived the experiments. A.S. and D.Z. analyzed data and wrote the manuscript. M.R., J.W. and H.A.H contributed to discussion and reviewed and edited the manuscript. D.Z. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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<th>Control (n = 75)</th>
<th>Diabetes (n = 96)</th>
<th>P value</th>
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<tr>
<td>Age (years)</td>
<td>61.0 (24.0)</td>
<td>58.0 (16.0)</td>
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<tr>
<td>Sex (% male)</td>
<td>69.3</td>
<td>67.7</td>
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<td>BMI (kg/m²)</td>
<td>24.3 (5.3)</td>
<td>31.4 (6.8)</td>
<td>&lt; 0.0001</td>
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<td>Height (cm)</td>
<td>176 (14)</td>
<td>174 (14)</td>
<td>0.099</td>
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<tr>
<td>Smoker (%)</td>
<td>16.2</td>
<td>29.0</td>
<td>0.053</td>
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<tr>
<td>Peroneal MNCV (m/s)†</td>
<td>46.0 (7.0)</td>
<td>44.0 (8.0)</td>
<td>0.001</td>
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<td>Sural SNCV (m/s)†</td>
<td>46.0 (5.4)</td>
<td>44.0 (9.5)</td>
<td>0.001</td>
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<td>Malleolar VPT (µm)†</td>
<td>0.94 (1.51)</td>
<td>1.19 (2.18)</td>
<td>0.001</td>
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<td>Warm TDT (°C)†</td>
<td>39.7 (7.0)</td>
<td>39.5 (6.8)</td>
<td>0.983</td>
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<td>Cold TDT (°C)†</td>
<td>29.1 (3.0)</td>
<td>28.4 (4.8)</td>
<td>0.028</td>
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<tr>
<td>Neuropathy Symptom Score (NSS)‡*</td>
<td>0.13 ± 0.83</td>
<td>0.90 ± 2.18</td>
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<tr>
<td>Neuropathy Disability Score (NDS)‡*</td>
<td>1.17 ± 1.70</td>
<td>1.45 ± 2.02</td>
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Data are expressed as median (IQR) except for sex and smoker variables which are expressed as (%). Values within parentheses represent interquartile range. T-test and Mann-Whitney-U-test were used where appropriate to calculate differences between groups. P < 0.05 was considered significant. * - Data are expressed as mean ±SD. † – Group comparisons were adjusted for age, sex, BMI and smoking. (Abbreviations: MNCV - motor nerve conduction velocity; SNCV - sensory nerve conduction velocity; VPT – vibration perception threshold; TDT – thermal detection threshold)
### Table 2–Multiple linear regression analysis for the relationship between LC density and M-value in women with type 2 diabetes

<table>
<thead>
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<th>β</th>
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<td>M-value</td>
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<td>Age</td>
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<td>BMI</td>
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<td>Smoking</td>
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<td>HbA1c</td>
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<td>0.892</td>
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$R^2=0.512$ for model
FIGURE LEGENDS

**Figure 1.** Intraepidermal nerve fiber density (IENFD) and Langerhans cell (LC) density in recently diagnosed type 2 diabetic patients and control subjects. Means of IENFD (A) and LC density (B) between the groups were compared using the *t*-test. Each spot represents one individual. The horizontal line represents the median value while the whiskers show the 25th and the 75th percentile. Representative images of skin sections from a healthy control with a high LC density (C) and a type 2 diabetes patient with a low LC density (D).

**Figure 2.** Sex specific association of M-value and Langerhans cell (LC) density in type 2 diabetic subjects. Association between M-value and LC density in women ($R^2=0.435$; $P<0.0001$) and men ($R^2=0.001$; $P=0.819$) was assessed using linear regression analysis.
Figure 2

A (Women) and B (Men) show scatter plots with M.Value on the y-axis and Langerhans cells (cells/mm²) on the x-axis. The plots indicate a linear relationship between M.Value and Langerhans cells for women, whereas the data for men is more scattered.