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Nerve-Langerhans cell interactions in diabetes and aging

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Summary. Cutaneous infections are a leading cause of hospitalization of diabetic patients. Langerhans cells (LCs) are antigen-presenting cutaneous dendritic cells that protect against infections, and effects of diabetes and aging on these cells are unclear. We examined LCs in footpads of rats with streptozotocin-induced diabetes at 3 months of age following 4 weeks of diabetes, and at 6 months following 16 weeks of diabetes. Immunostaining of LCs using the selective marker protein langerin showed cutaneous LC composition increased between 3 and 6 months of age owing to increased LC numbers and size in control rats. In diabetic rats, LC numbers increased with age but, unlike 6 month old controls, cell size did not, suggesting that diabetes impairs the increase in cell size that is a hallmark of LC maturation. Diabetes reduced LC numbers after 4 weeks and numbers and sizes following 16 weeks. We examined the relation between LC and innervation and found that, while axon density decreased with aging, it was not affected by 16 weeks of diabetes. However, LCs expressing the neuronal marker PGP9.5 represented a source of error in axonal counts. These findings support the hypothesis that diabetes substantially impacts LC proliferation and maturation independent of effects on cutaneous innervation. Accordingly, the interactions of diabetes and aging on LCs may be important factors in predisposing diabetic patients to cutaneous ulcers and infections.

Key words: Langerhans cell, Diabetes, Intraepidermal nerve fibers, Aging, Langerin

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Introduction

Infection of cutaneous ulcers is a major cause for hospitalization and amputation among the diabetic population (Reiber et al., 1998; Cruciani et al., 2009; Xie et al., 2010). Although the incidence of cutaneous infections is not greater (Oumeish, 2008), healing time is delayed and morbidity more severe (Ferringer and Miller, 2002; Xie et al., 2010). The reasons for the greater frequency of ulcer pathologies in diabetes is unclear, but accompanying neuropathies and angiopathies may well contribute to delayed healing (Meijer et al., 2001; Wohlrab et al., 2007; Ghanassia et al., 2008; Lauterbach et al., 2010). In addition, the skin contains an intrinsic immune system that helps protect against infection. Antigen presenting cells (APCs) include macrophages, B cells, and dendritic cells, and are critical for preventing foreign antigen infiltration. Impairments in the cutaneous immune system could therefore contribute to poor outcomes in diabetics developing cutaneous ulcers.

The main APC in the epidermis is the Langerhans cell (LC). LCs are stellate dendritic cells derived from hematopoietic precursors (Merad et al., 2002), and are also found within stratified squamous epithelium in other body regions (Pieri et al., 2001; Merad et al., 2008) including the oral cavity (Rowden, 1981), esophagus (Rowden, 1967), and vagina (Iijima et al., 2007). LCs express antigen-presenting proteins such as major histocompatibility complex (MHC)- I and II, human leukocyte antigen-DR (HLA-DR), and CD1a (Harrist et al., 1983; Ayala-Garcia et al., 2005; Mutyambizi et al., 2009; Romani et al., 2010), and their primary function is believed to be to present atypical self or foreign antigens to T cells (Merad et al., 2008; Zaba et al., 2009). LCs are also characterized by the presence of langerin, a type II Ca^{2+}-dependent lectin (Valladeau et al., 1999; Valladeau et al., 2000; Romani et al., 2010). Langerin has a

carbohydrate recognition domain that specifically binds to and is involved in uptake of mannose-containing antigens (Valladeau et al., 2000; Chatwell et al., 2008). The primary function of langerin is thought to be ligand internalization associated with Birbeck granule formation, a feature exclusive to epidermal LCs (Valladeau et al., 1999, 2000; Kissenpfennig et al., 2005). Consequently, these cells are important in cutaneous immune responses (Mutyambizi et al., 2009; Romani et al., 2010).

Diabetes could influence LCs through direct effects on cell maturation or longevity, or indirectly through microvascular changes (Jeffcoate and Harding, 2003; Wohlrab et al., 2007) or altered keratinocyte growth factor production. Further, intraepidermal nerve fibers (IENFs) appear to regulate LC composition, as peripheral degeneration increases LC numbers (Stankovic et al., 1999; Hsieh et al., 1996; Lindenlaub and Sommer, 2002; Lauria et al., 2005a,b; Siau et al., 2006; Jin et al., 2008). Since neuropathic changes are common in diabetes (Lauria et al., 2005a,b; Lauria and Lombardi, 2007; Sommer, 2008; Beiswenger et al., 2008; Nebuchennykh et al., 2009), it is important to assess whether changes in IENFs may be a factor mediating diabetes' effect on LCs.

Thus far, two studies have quantified effects of diabetes on LCs, yielding conflicting results (Ziegler and Standl, 1988; Lauria et al., 2005a,b). These studies examined skin from different locations, species, and at different times after the onset of diabetes using either MHC-II or protein gene product 9.5 (PGP9.5) as a LC marker. PGP9.5 is expressed by LCs (Hsieh et al., 1996; Stankovic et al., 1999; Lin et al., 2001; Lauria et al., 2005a,b; Beiswenger et al., 2008; Jin et al., 2008), but is also present within IENFs and commonly used to quantify innervation (Jackson and Thompson, 1981; Lauria et al., 2005a,b; Lauria and Lombardi, 2007; Beiswenger et al., 2008; Sommer, 2008; Lauria et al., 2009; Nebuchennykh et al., 2009). Since LCs form intimate associations with IENFs, questions have emerged regarding whether PGP9.5-immunoreactive (ir) LCs may be a confounding factor in quantifying IENFs. In contrast, langerin appears to be a far more selective marker, at least in mice and humans (Valladeau et al., 1999; Stoitzner et al., 2003), although reports using langerin in rats are limited (Meyer et al., 2010).

The purpose of this study was to systematically examine effects of different durations of streptozotocin-induced diabetes and, concurrently, age on epidermal LCs and innervation using dual staining for PGP9.5 and langerin. Our objective was to identify cellular mechanisms that could contribute to the profound incidence and severity of cutaneous ulcers and infections in diabetic patients.

Materials and methods

All animal protocols were approved by the University of Kansas Medical Center's Animals Care and Use Committee, and were in accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

Sixty-day old female Sprague Dawley rats (Harlan Laboratories, Inc., Indianapolis, IN) were anesthetized (60 mg/kg ketamine HCL, Ketaject, 0.4 mg/kg atropine sulfate, and 8 mg/kg xylazine Xyla-Ject, intraperitoneal (ip) injection) and bilaterally ovariectomized under aseptic conditions (Blacklock and Smith, 2004). Estrogen is known to alter LC numbers (Koyama et al., 1987; Hernandez-Segura et al., 2005; Kovats and Carreras, 2008), and reproductive hormone levels vary with age and reproductive status in both males in females. Therefore, using females with constant and very low levels of estrogen after ovariectomy (Strom et al., 2008) eliminates a significant confounding variable.

Diabetes induction

One week following ovariectomy, blood samples were taken from the tail vein and blood glucose measured with a TrueTrack Smart System glucometer (Ft. Lauderdale, FL). Animals then received a single ip injection of streptozotocin (STZ, 60 mg/kg, in 10 mM citrate buffer with 0.9% NaCl, pH4.5, (n=17) or vehicle buffer (n=11). Three days following STZ administration, blood glucose levels were again assessed, and animals with levels ≥270mg/dl were considered diabetic and included in the experiment. Animals were maintained for 4 (n=6) or 16 weeks (n=11) at which time, blood glucose and weight were measured.

Immunostaining and confocal imaging

Animals were anesthetized with Beuthanasia (195 mg/kg Sodium Pentobarbital, 25 mg/kg Phenytoin Sodium, ip; Schering-Plough Animal Health Corp., Union, NJ) and footpads were harvested. Tissue was immediately immersed in Zamboni fixative for 24-hours. Tissue was rinsed daily in 0.1 M phosphate buffered saline (PBS) for 10-14 days. After rinsing, footpads were immersed in 30% sucrose solution. Tissue was frozen in tissue freezing medium (Electron Microscopy Sciences, Hatfield, PA) and stored at -80°C. Footpads were serially sectioned at 18 μm thickness perpendicular to the surface and along the length of the footpad. Three sections spaced 342 μm were analyzed per animal.

Sections were stained with goat anti-langerin antiserum (1:100, Santa Cruz Biotechnology, Santa Cruz, CA,) to identify epidermal LCs. Immuno-fluorescence with the langerin antibody in tissue from mice expressing enhanced green fluorescent protein under the control of the langerin gene (kindly provided by Dr. Bernard Malissen) confirmed that our langerin antibody was specific for Langerhans cells. Also, sections were stained with a rabbit anti-human PGP9.5 antibody (1:600, AbD Serotec, Oxford, UK,) to identify cutaneous nerve fibers. Western blots of dorsal root ganglion protein immunostained with PGP9.5 antibody

showed a band of 27 kDa, serving as a positive control (Doran et al., 1983). Sections were blocked with donkey serum followed by overnight incubation with primary antibodies, rinsing in PBS with 3% triton for 30 minutes, and incubation for one hour with secondary antibodies (Cy3 Donkey anti-Goat and Alexa 488 Donkey anti-Rabbit; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Sections were rinsed in PBS with 3% triton and mounted with Fluoromount G. Images were taken with 60x or 100x oil immersion objectives on an Eclipse 90i microscope equipped with a Nikon C1si confocal system and a D-Eclipse camera (Melville, NY). 2-D images were created using maximum projection on volume renderings of Z-stacks with Nikon NIS-elements Advance Research 3.1 software (Melville, NY).

Stereological analysis of LCs

For each animal, three sample fields (0.148 mm^2/field) per section were obtained with a 20X objective using a Photometrics Cool SNAP-EZ Camera (Roper Scientific Inc., Tucson, AZ) and Nikon Eclipse TE300 inverted microscope, and analyzed (MetaMorph, Molecular Devices, Downingtown, PA). Epidermal area within the sample field was estimated by tracing around the stratum granulosum and basal cell layers while excluding the stratum corneum. Numbers of cells positive for langerin immunoreactivity (+) were counted. The contribution of langerin+ cells to the overall epidermal sample area was obtained by superimposing a stereological grid (13.23 μm intersections), counting numbers of intersects overlying LCs, and multiplying by the grid square area corresponding to each intersection (175 μm^2), using approaches similar to previous studies (Zoubina et al., 1998; Blacklock and Smith, 2004; Blacklock et al., 2004). The apparent percentage of epidermal area occupied by langerin+ cells was also calculated by dividing total langerin area by epidermal area and then multiplying by 100. To obtain an index of overall LC size, total langerin+ area was divided by the number of langerin+ cells.

Quantitation of IENFs

A method originally defined by Kennedy et al. (2005) and adopted by European Federation of Neurological Societies (EFNS) (Lauria et al., 2005a,b) was used to quantify epithelial innervation. Individual IENFs immunostained for PGP9.5 that cross the dermal-epidermal junction were counted while excluding any secondary branching. Counts were divided by the length of the epidermis and expressed as IENF/mm. Individual IENFs were visually counted at three regions along the length (distal, middle, and proximal) of each footpad section with a 40x objective and 10x eyepiece on a Nikon Eclipse TE300 inverted microscope (Nikon Corp., Tokyo, Japan). Each region spanned 0.42 mm. To determine epidermal length, the basement membrane was measured. For each region, IENF counts were divided by epidermal length to obtain PGP9.5+ IENF/mm. We also repeated these counts in samples double stained for langerin, in which PGP9.5+ projections co-labeled with langerin were excluded from counts. These data are expressed as PGP9.5+/langerin- IENF/mm.

Statistical Analysis

Weight and blood glucose levels were analyzed with two way repeated measures analysis of variance and Student-Newman-Keuls post-hoc analysis. Differences in epidermal LC density, size, and percent epidermal area were compared by t-test or two way analysis of variance and Student-Newman-Keuls post-hoc analysis. IENF/mm data were compared by t-test with Bonferroni correction. The effect of age on nerve density was analyzed with two way analysis of variance. Differences were considered significant if p value was <0.05. Data are expressed as Mean ± Standard Error Mean (SEM).

Results

Blood glucose and body weight

Diabetic status at 4 and 16 weeks following STZ injections was confirmed by measuring blood glucose levels (Table 1). Vehicle-injected rats remained euglycemic, while glucose levels of STZ-treated rats were significantly greater than aged-matched controls ($p<0.001$ and $p<0.001$, respectively). Moreover, glucose levels for STZ-treated rats at 16 weeks were higher than levels at 4 weeks ($p<0.001$). Body weight within groups was not affected by age but was reduced after 4 and 16 weeks of diabetes ($p<0.001$,Table 1).

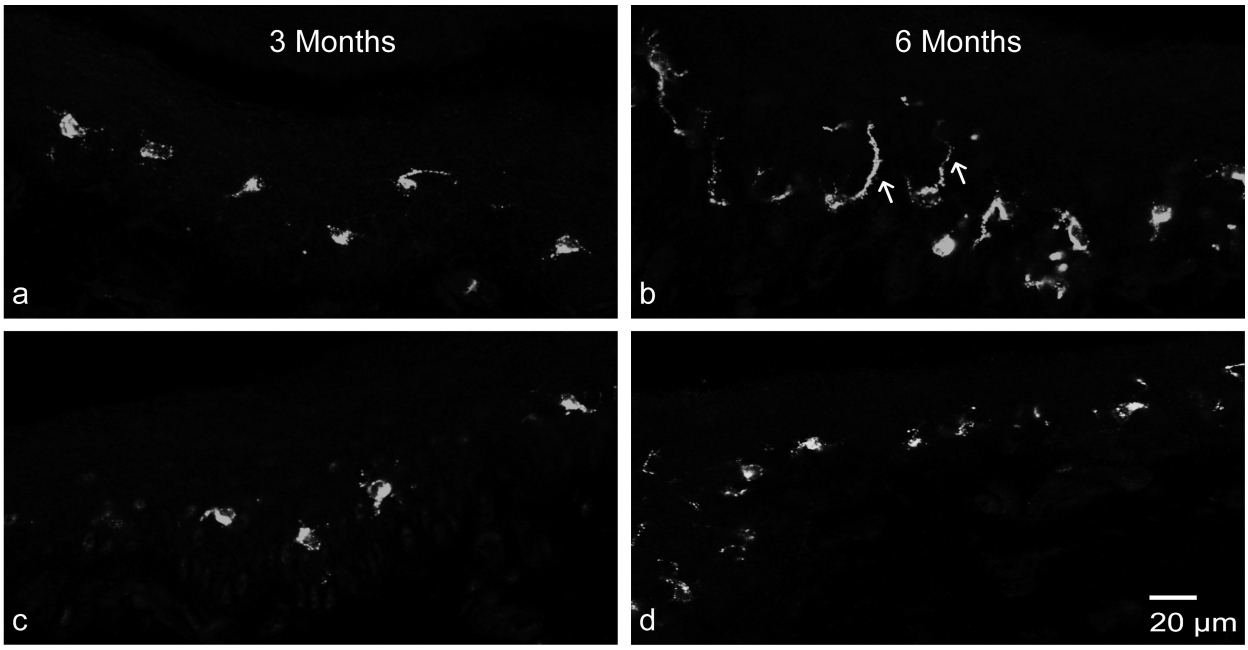
Langerin immunostaining in euglycemic rats at 3 months of age

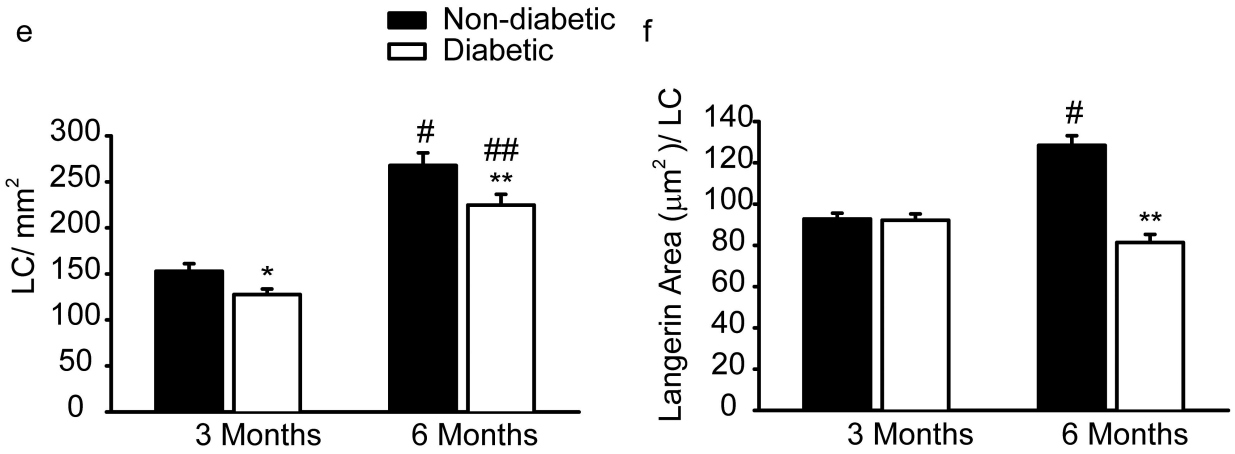
Immunostained footpad sections from 3-month-old rats 4 weeks following vehicle injection revealed a langerin+ epidermal cell population. Langerin+ cells were primarily found within the stratum spinosum,

Table 1. Blood glucose levels and body weight at end of experiment.

	Glucose (mg/dL)	Weight (grams)
3 months		
Non-diabetic	91±5	282±8
Diabetes, 4 wks	408±34*	233±10*
6 months		
Non-diabetic	120±9	306±4
Diabetes, 16 wks	529±22*	219±11*

Blood glucose levels were significantly higher than controls after 4 and 16 weeks of diabetes. Glucose levels were significantly greater after 16 weeks of diabetes when compared to 4 weeks of diabetes. Body weight significantly decreased after four and 16 weeks of diabetes. Data is presented as mean ± SEM. *: $p<0.001$.





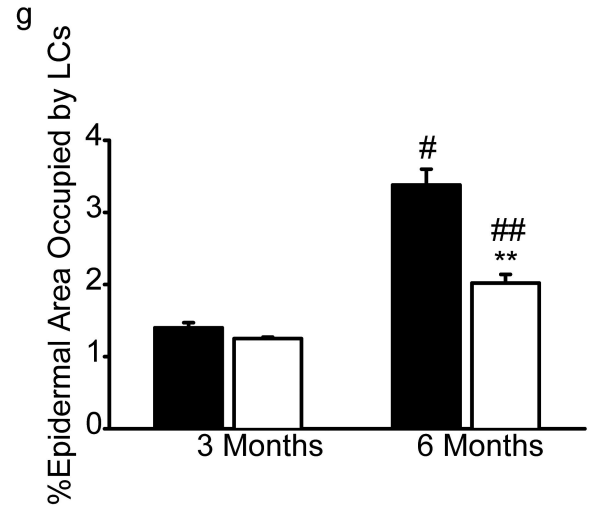
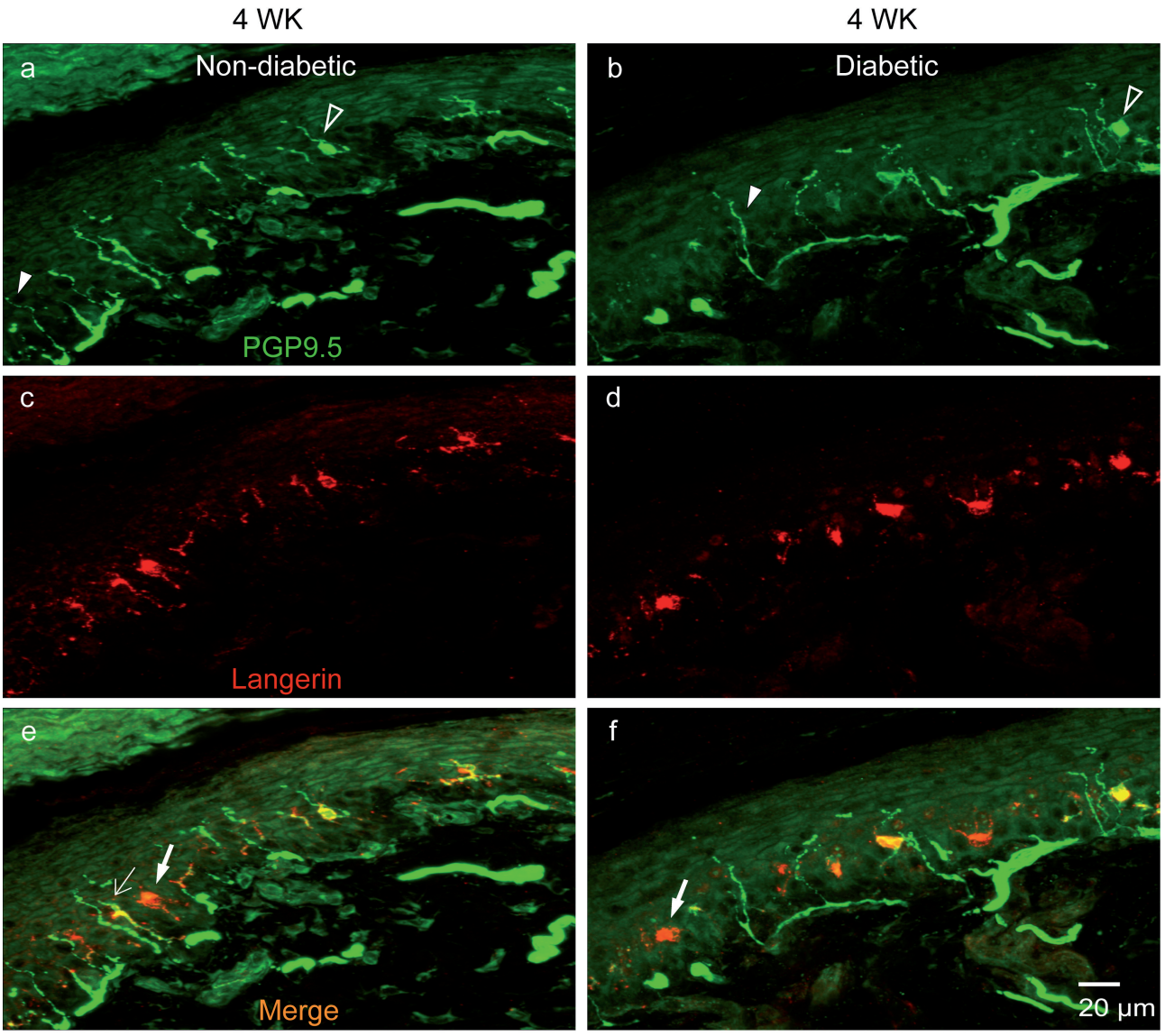


Fig. 1. Quantiative Analysis of Langerin+ LCs in the Rat Footpad. The effect of age on LCs was quantified by comparing three month old non-diabetic **(a)** and diabetic animals **(c)** to six month old non-diabetic **(b)** and diabetic animals **(d)**, respectively. The effect of STZ treatment on LCs was quantified by comparing non-diabetic animals at four and 16 weeks post-treatment (**a and b**, respectively) to diabetic animals at four and 16 weeks post-treatment (**c and d**, respectively). White arrows reveal longer processes on LCs in six month old non-diabetic animals. Bar graphs reflect LC density **(e)**, size **(f)**, and the % of epidermal area occupied by LCs **(g)** at 3 (4 weeks post-STZ) and 6 months (16 weeks post-STZ) of age. *: p=0.032 when compared to 4wk non-diabetic animals, #: p<0.001 when compared to 4 week non-diabetic animals, ##: p<0.001 when compared to 4 week diabetic animals, **: p<0.001 when compared to 16 week non-diabetic animals.



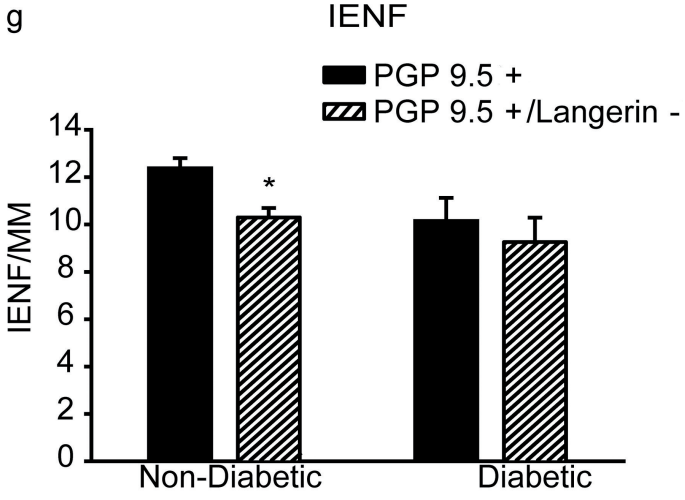
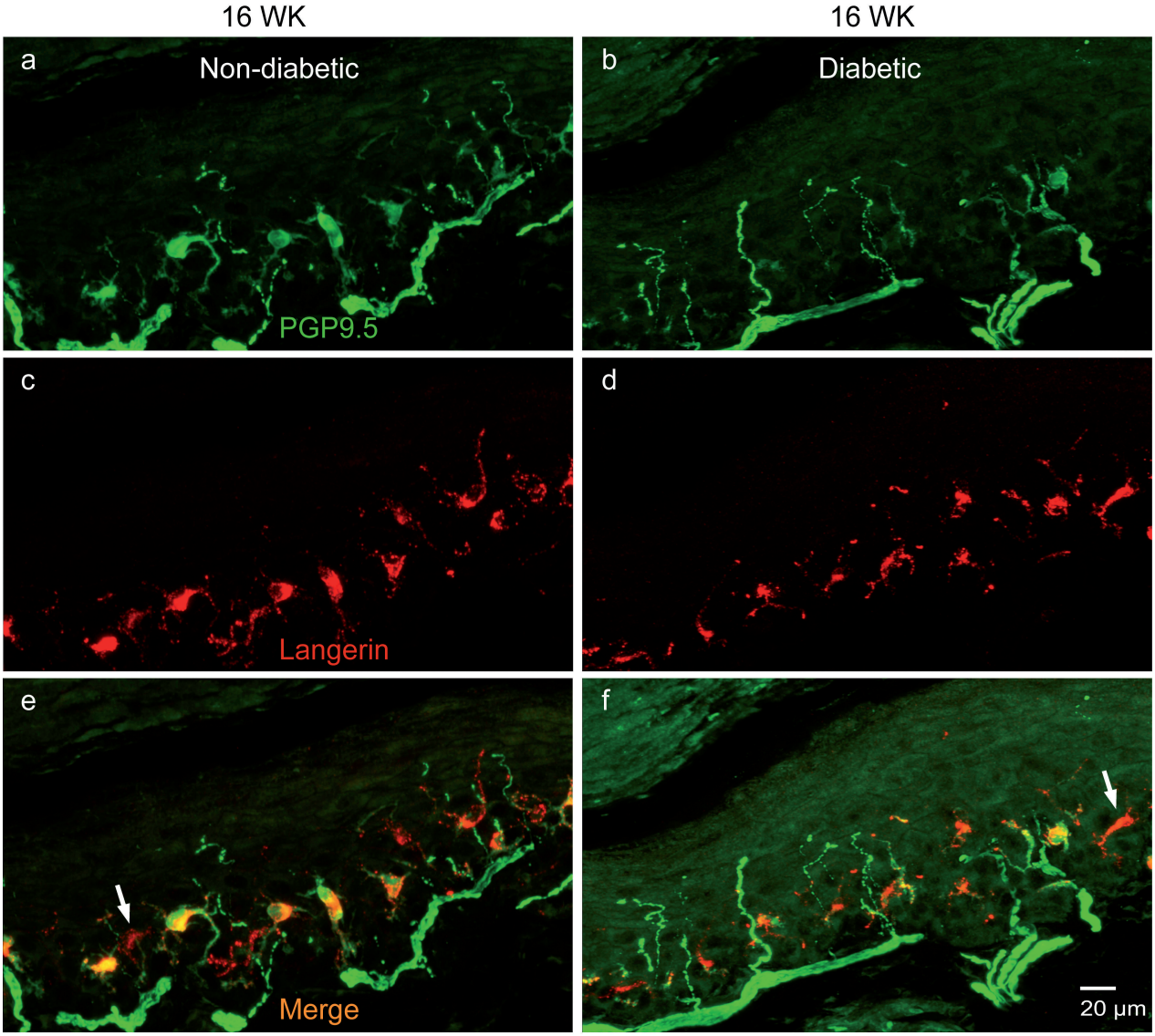


Fig. 2. LC contribution to IENF values at 4 Weeks of diabetes. Footpad sections were double-stained with a PGP9.5 (**a and b**) and langerin (**c and d**) antibody to identify IENFs and LCs, respectively. PGP9.5 and langerin images were merged (e and f) to display LC contribution to PGP9.5(+) IENFs in non-diabetic (**a, c, and e)** and diabetic animals (**b, d and f**) at four weeks post-treatment. Open arrowhead designates PGP9.5+ LCs. Filled arrowheads mark an IENF that branches from dermal nerve bundle and enters epidermis. Thin arrow indicates PGP9.5+/Langerin+ that would be considered a PGP9.5+ IENF if langerin immunoreactivity was not considered. Closed arrow reflects PGP9.5-/Langerin+ LCs. Before langerin immunoreactivity was considered, IENF density was quantified (solid bars). After PGP9.5+ profiles that express langerin were excluded, PGP9.5+/langerin- IENFs were calculated (dashed bars). *: p=0.024 as compared to non-diabetic PGP9.5+ IENF density.



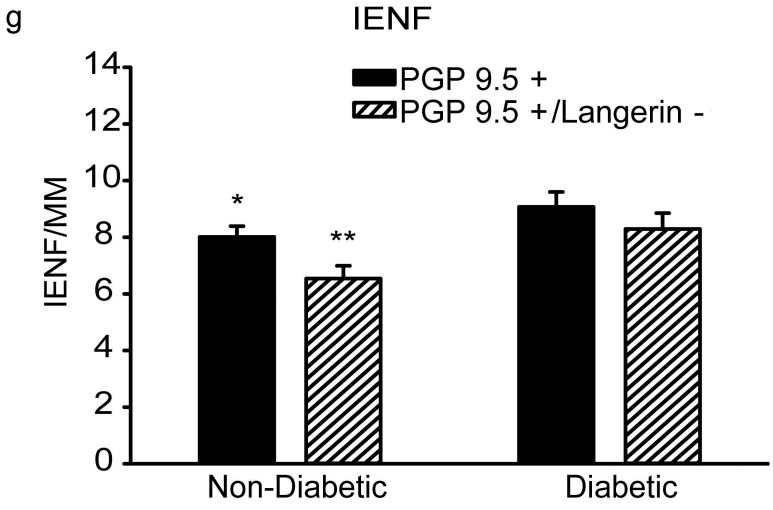


Fig. 3. LC Contribution to IENF Values at 16 Weeks of diabetes. Footpad sections from non-diabetic animals **(a, c and d)** and diabetic animals (**b, d and f**) were double-stained with PGP9.5 (**a and b**) and langerin (c and d) antibodies. Merged images (**e and f)** reveal LC contribution to IENF density by means of PGP9.5 expression. Closed arrow reflects PGP9.5-/Langerin+ LCs. Before langerin immunoreactivity was considered, IENF density was quantified (solid bars). After PGP9.5+ profiles that express langerin were excluded, PGP9.5+/langerin- IENFs were calculated (dashed bars). *: $p<0.001$when compared to PGP9.5+ IENF density for 3 month old non-diabetic rats and **: $p=0.005$ when compared to PGP9.5+/langerin- IENF density for 3 month old non-diabetic rats

granulosum and basal layers (Fig. 1a), and occasionally in the stratum corneum and below the basement membrane (not shown). Langerin+ cells were stellate or spindle-shaped with extensive processes, sometimes stretching across all epidermal layers except the stratum corneum. Langerin immunoreactivity appeared to be intra-cytoplasmic and punctate, localizing mainly to the soma with less accumulation in distal-most processes (Fig. 1a).

Langerin immunostaining in euglycemic rats at 6 months of age

Quantitative analysis showed a 75% increase in LC density in non-diabetic animals when compared to 3 month old non-diabetic animals, ($p<0.001$, Fig. 1b,e). Moreover, LC size was 38% greater at 6 months as compared to rats at 3 month of age ($p<0.001$, Fig.1b,f). Overall, epidermal area occupied by LCs increased by 141% between 3 and 6 months of age ($p<0.001$, Fig. 1g).

Langerin immunostaining in hyperglycemic rats at 3 and 6 months of age

Rats at 3 months of age that received a single ip injection of STZ 4 weeks earlier appeared to have fewer LCs when compared to 3 month old euglycemic rats (Fig. 1c), and quantitative analysis confirmed a 17% decrease ($p=0.032$, Fig. 1e). However, 4 weeks of diabetes did not alter LC size (Fig. 1f) or epidermal area occupied by LCs (Fig. 1g).

Rats aged 6 months rendered diabetic for 16 weeks displayed numerous small LCs in their epidermis (Fig. 1d). Relative to younger diabetic rats, LCs were increased 76% ($p<0.001$, Fig. 1e). However, the age-related increase that occurred in non-diabetic rats was attenuated by diabetes ($p=0.041$, Fig. 1e). In addition, LC size showed no age-related increase between 3 and 6 months in diabetic animals (Fig. 1f). When compared to aged controls, LC size decreased by 37% after 16 weeks of diabetes ($p<0.001$, Fig. 1f). The overall percentage of epidermal area occupied by LCs increased by 61% between 3 and 6 months, but remained markedly lower than that of euglycemic rats at 6 months ($p<0.001$, Fig. 1g).

Intraepidermal nerve fibers

Footpad sections double-stained for PGP9.5 and langerin were examined to determine how age and diabetes affect IENFs and their relationship to LCs. PGP9.5 immunostaining revealed large nerve bundles running parallel to the basement membrane. Smaller branches emanated from the bundles and penetrated the epidermis perpendicularly (Figs. 2a,b, 3a,b). Staining also showed PGP9.5 immunoreactivity within the cytoplasm of stellate and spindle-shaped cells within non-keratinized epidermis (Figs. 2a,b, 3a,b). These cells frequently displayed long processes oriented perpendicularly to the epidermal longitudinal axis. Co-localization of PGP9.5 and langerin revealed stellate and spindle-shaped cells were LCs (Figs. 2e,f, 3e, f).

Confocal images revealed PGP9.5+ fibers that could be traced to subepidermal nerve bundles frequently showed intimate associations with langerin+ LCs; nerve fibers were also frequently intermingled between the fine processes of LCs (Figs. 2e,f, 3e,f). In fact, in PGP9.5 immunostained specimens, it was often impossible to distinguish with certainty which 'fibers' were axons and which were LC projections.

To determine whether LC projections contribute to counting errors that may occur when quantifying epidermal innervation, we counted IENFs in sections costained for PGP9.5 and langerin. PGP9.5+ LC processes represented 15-19% of overall IENF counts for non-diabetic rats at both 3 (Fig. 2g) and 6 months of age (Fig. 3g), but achieved statistical significance only at 3 months ($p=0.024$, Fig. 2g). LC contributions to counts in diabetic groups were more modest and not statistically significant (Figs. 2g, 3g).

In non-diabetic rats, we detected a decrease in numbers of both corrected (i.e., LC processes excluded, $p=0.005$) and uncorrected ($p<0.001$) IENF counts between 3 and 6 months of age (Figs. 2g, 3g). There were no differences in corrected and uncorrected IENF counts between 3 and 6 month old diabetic rats. Irrespective of whether IENF counts were corrected for LC contributions, there was no significant difference between diabetic and non-diabetic rats at 4 and 16 weeks after induction (Figs. 2g, 3g).

Discussion

Increase in LC density between 3 and 6 months in non-diabetic rats is consistent with greater LC proliferation (i.e. more cell migration or replication). Similarly, increased LC size in older rats is consistent with greater functional maturity (Larsen et al., 1990; Nishibu et al., 2006). Collectively, these changes resulted in an overall increase in LC contribution to foot pad skin, implying that LCs may be able to survey a greater volume of foot epidermis in older animals. Changes in LC status and cell function are not unexpected, given the increasing cumulative exposure to environmental antigens with time.

Age-related changes in epidermal LC composition may vary as a function of species and location. The rat foot pad represents the main point of physical contact with the external environment and therefore is constantly exposed to potential antigens. In contrast, studies in mice and humans suggest that other sites may not undergo comparable increases; for example, skin samples from ear and buttocks showed evidence of decreased LC composition with age (Bhushan et al., 2002; Cumberbatch et al., 2002; Agrawal et al., 2007). Varied findings in age-related LC changes may be influenced by differences in species (Breathnach, 1991), skin type (e.g., glabrous vs. hairy), and location (Berman et al., 1983; Thomas et al., 1984). Nonetheless, the foot is of particular clinical relevance because it is frequently

affected by cutaneous ulceration and infection in diabetic patients, often resulting in amputation.

Diabetes resulted in reduced hind paw LC density, but did not affect size or percentage of epidermal area occupied by LCs at 4 weeks post-treatment. These results suggest that in the early stages, diabetes selectively reduces LC numbers, presumably by reducing proliferation. Despite this reduction, the overall contribution of LCs to foot pad cutaneous composition remained relatively normal. Although functional studies are necessary to determine whether a normal immune response is retained, these findings imply that area of cutaneous tissue surveyed by LCs remains relatively normal in early STZ-induced diabetes.

In contrast, rats enduring 16 weeks of hyperglycemia showed decreases in LC density, size and the percentage of epidermal area occupied by LCs. This suggests that a longer duration of hyperglycemia may impair both proliferation and maturation. These findings are in agreement with those of Zeigler and Standl (1988) who found a reduction in LCs in early Type I diabetes in humans but differ from a report of increased LCs in the STZ diabetic rat (Lauria et al., 2005a,b). As noted above, differences may reflect regional variations, but may also represent differences in quantitative methods (correcting for length only vs. epidermal volume), and in LC markers (langerin vs. PGP9.5, which does not label all LCs). In any event, our study shows diabetes can have a negative effect on LC proliferation and maturation, which may lead to reduced epidermal antigen surveillance.

As in euglycemic rats, the percentage of epidermal area occupied by LCs in diabetic rats at 6 months of age was increased relative to that at 3 months, although the increase was attenuated. This was due to combined effects on LC proliferation and maturation, as diabetes diminished both cell density and size. Thus, duration of diabetes appears to affect LC dynamics negatively. It is unclear whether age of onset is also a factor, but in any event, the effects of diabetes were certainly more pronounced in older rats.

Because previous reports suggested that changes in epidermal innervation can alter LC density, it was important to determine if innervation was affected in our STZ-diabetic rats. However, because LCs express PGP 9.5 and are often intimately associated with axons, we were concerned that fine PGP 9.5+ processes from LCs could contribute to IENF counts. While most LCs expressed both langerin and PGP 9.5, a significant proportion of LCs expressed langerin but not PGP 9.5. Variation in PGP+ LCs has been reported previously, but only in association with phenotypic changes occurring with loss of cutaneous innervation (Hsieh et al., 1996).

'Axon' counts in material double-stained for langerin revealed that PGP 9.5+ LC processes may be a potential source of error. While this occurred in all specimens, the contribution was greatest within non-diabetic animals. We attribute this to the findings that diabetes reduces the number, size and complexity of LCs. These results show that PGP9.5+ within LC processes can be a source of error in IENF counts if they are not excluded by co-staining with a more selective LC marker such as langerin.

Our findings that IENF counts between 3 and 6 months were reduced in non-diabetic rats, whether or not they were corrected for contributions PGP 9.5+ LC processes, agree with those of others who also report a decrease in with age (Goransson et al., 2004; Lauria et al., 2010). In contrast, there was no clear age-related decline in IENF density in diabetic rats. After 4 weeks of diabetes, rats did not display a reduction in IENFs which is in accord with a prior report failing to detect axon reductions (Karanth et al., 1990). However, the finding that IENF counts were not decreased following 16 weeks of diabetes was somewhat unexpected, as other studies have reported reductions after 12 or more weeks (Lauria et al., 2005a,b; Toth et al., 2006; Leonelli et al., 2007; Roglio et al., 2007; Jin et al., 2009). Because our rats showed all the classical signs of insulin-deficient diabetes including hyperglycemia, weight loss, polydipsia and polyuria, the difference is unlikely to be attributable to ineffective STZ treatment. Other factors may include age at which diabetes is induced, method of induction, and sampling and quantitation approaches. Additionally, we cannot rule out the possibility that changes in parent axon structure or conduction properties occurred that were not detected. In any case, the absence of significant loss of cutaneous nerve fibers supports the idea LC changes in diabetes are not secondary to outright nerve degeneration.

It is unclear how diabetes alters LC dynamics. One mechanism may involve altered production of granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is necessary for LC proliferation and maturation (Heufler et al., 1988; Kingston et al., 2009). Diabetes reduces GM-CSF production in wounds by 50% (Fang et al., 2010), and delayed wound healing in diabetes is reversed by GM-CSF administration (Fang et al., 2010). Therefore, diminutions in GM-CSF may impair recruitment and maturation of LCs in diabetes.

Collectively, this study shows that LCs are strongly influenced by age and diabetes. Given that both age and diabetes can be predisposing factors in development of cutaneous infections and ulcerations, our findings suggest that effects on dendritic cells may well play a role in the development of these pathologies.

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