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Langerhans cells regulate cutaneous innervation density and mechanical sensitivity in mouse footpad

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HIGHLIGHTS

- Depletion of cutaneous Langerhans cell reduces numbers of sensory axons.
- Langerhans cell depletion reduces epidermal NGF and GDNF mRNA.
- Mechanical sensitivity is increased following Langerhans cell depletion.
- Langerhans cells therefore regulate cutaneous innervation density and sensitivity.

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ABSTRACT

Langerhans cells are epidermal dendritic cells responsible for antigen presentation during an immune response. Langerhans cells associate intimately with epidermal sensory axons. While there is evidence that Langerhans cells may produce neurotrophic factors, a role in regulating cutaneous innervation has not been established. We used genetically engineered mice in which the diphtheria toxin (DT) receptor is targeted to Langerhans cells (Lang-DTR mice) to assess sensory axon–dendritic cell interactions. Diphtheria toxin administration to wild type mice did not affect epidermal structure, Langerhans cell content, or innervation density. A DT administration regimen supramaximal for completely ablating epidermal Langerhans cells in Lang-DTR mice reduced PGP 9.5-immunoreactive total innervation and calcitonin gene related peptide-immunoreactive peptidergic nociceptor innervation. Quantitative real-time polymerase chain reaction showed that epidermal gene expression of brain derived neurotrophic factor was unchanged, but nerve growth factor and glial cell line-derived neurotrophic factor mRNAs were reduced. Behavioral testing showed that, while thermal sensitivity was unaffected, mice depleted of Langerhans cells displayed mechanical hypersensitivity. These findings provide evidence that Langerhans cells play an important role in determining cutaneous sensory innervation density and mechanical sensitivity. This may involve alterations in neurotrophin production by Langerhans or other epidermal cells, which in turn may affect mechanical sensitivity directly or as a result of neuropathic changes.

1. Introduction

Intraepidermal nerve fibers (IENFs) represent terminations of dorsal root ganglion sensory axons [17]. They are critical for identifying tactile, thermal, and noxious stimuli [17,19] and in mounting local inflammatory responses [31]. Accordingly, IENFs are critical components of skin and their loss in conditions such as diabetes [13] and Guillain–Barré syndrome [25] can contribute substantially to pathology phenotypes.

Mechanisms responsible for establishing and maintaining cutaneous innervation remain unclear. Keratinocytes, the predominant epidermal cell type, are known to produce neurotrophic factors

Abbreviations: BDNF, brain derived neurotrophic factor; CGRP, calcitonin gene-related peptide; DT, diphtheria toxin; GDNF, glial cell line derived neurotrophic factor; IENF, intraepithelial nerve fiber; -ir, immunoreactivity; LC, Langerhans cell; NGF, nerve growth factor; NTF, neurotrophic factor; PGP9.5, protein gene product 9.5.

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(NTFs) [21,23,33]. However, melanocytes also synthesize neurotrophic proteins [20]. Similarly, Langerhans cells (LC) represent a substantial component of the skin [6] and have also been shown to produce NTFs [34]. Therefore, cells in addition to keratinocytes may be important in regulating epidermal innervation.

Langerhans cells have a well-established role in immunity. As the primary resident epidermal antigen-presenting cell, they capture and translocate foreign antigens to lymph node T cells, thus participating in innate and adaptive immune responses [2,4]. LCs are distinguishable from other cutaneous and neural cells by their expression of langerin, a protein involved in ligand internalization and antigen presentation [36].

Despite their role in immunity, LCs have long been suspected to have neurally-related functions, a concept going back to their initial description [14]. Subsequent studies show that LCs make intimate contacts with IENFs [6–8], and LC density is apparently regulated by cutaneous innervation [9,28,30]. However, a role of LCs in regulating epidermal innervation has not been explored. The availability of genetically engineered mice where LCs can be selectively ablated provides an opportunity to explore the relationship between LCs and cutaneous innervation more thoroughly.

2. Materials and methods

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

2.1. Langerhan cell depletion

Lang-DTR mice (Lang-DTREGFP, a kind gift from Dr. Bernard Malissen, Centre d'Immunologie de Marseille Luminy) are C57BL/6 mice genetically engineered to express the human diphtheria toxin receptor (DTR) fused to enhanced green fluorescent protein under control of the langerin gene [11]. Administration of diphtheria toxin (DT) at concentrations not toxic to wild type mice produces selective LC depletion [11].

LCs were depleted beginning at 1 month of age by intraperitoneal injections of DT (1 μg in 100 μl, Sigma Aldrich, St. Louis, MO, $n = 5$) or vehicle (water, $n = 5$) on d0 (first injection), 4, 8, and 12 to Lang-DTR mice (see Fig. 4 for an experimental time line); this regimen is supramaximal for depleting epidermal LCs but is necessary to ensure complete and sustained elimination of all cutaneous langerin-ir cells [11]. To exclude effects that DT may have independent of LC depletion, we administered saline ($n = 6$) or DT at the same dosage ($n = 6$) to C57BL/6 mice (Jackson Laboratories).

Two days following the final DT injection, mice were anesthetized with isoflurane (Abbott) and right hind paws excised and fixed in Zamboni's solution for 24 h, rinsed in PBS for 14 d, immersed in 30% sucrose for 3–5 d, frozen in tissue freezing medium (Electron Microscopy Sciences), and sectioned serially at 20 μm in sagittal orientation. Left hind paws were removed for real time quantitative RT-PCR.

2.2. Immunohistochemistry

Sections were treated with 100 mM glycine in PBS for 30 min, rinsed and immersed for 1 h in SuperBlock buffer (ThermoScientific), and stained overnight with primary antisera to protein gene product 9.5 (PGP9.5, 1:800, rabbit IgG, AbD Serotec), calcitonin gene-related peptide (CGRP, 1:600, sheep IgG, Enzo Life Sciences), and langerin (1:800, goat IgG, Santa Cruz Biotechnology), which have been characterized previously [6,32]. After rinsing in PBST, sections were incubated for 1 h with Cy2-conjugated donkey anti-goat (1:200), Cy3-conjugated donkey anti-rabbit (1:800), or Cy3-conjugated donkey anti-sheep (1:400) IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.), rinsed in PBS and coverslipped with Fluoromount G. Three sections at 240 μm intervals were analyzed per animal.

2.3. Langerhans cell analysis

Images of langerin immunostained cells were captured from distal, middle, and proximal regions in each section with a 40× oil immersion objective on a Nikon Eclipse 90i microscope. LC density was measured by dividing numbers of LCs by epidermal area (μm^2) measured planimetrically, excluding the stratum corneum. Langerin immunostaining was used because GFP fluorescence can be attenuated by fixation and to allow direct comparison of LC features in Lang-DTR and wildtype mice. We evaluated LC size, an indicator of functional maturity [24], by dividing the langerin-ir area (μm^2) measured by thresholding, by numbers of LCs. To determine the percentage of epidermal area occupied by LCs, langerin-ir area was divided by total epidermal area and multiplied by 100.

2.4. Epidermal nerve fiber quantification

PGP9.5, a pan-neuronal marker [37], was used to identify all IENFs and CGRP to identify 'peptidergic' axons [35]. Three regions 0.42 mm in length spaced equidistantly along each section were selected to provide indices of innervation at the distal, middle, and proximal regions of the footpad. Individual IENFs crossing the dermal-epidermal junction were counted, excluding secondary branching [15]. Counts were divided by epidermal length and expressed as IENF/mm. Epidermal thickness was measured at the center of each image.

2.5. Neurotrophin mRNA expression in epithelium after Langerhans cell depletion

Left hind footpads were excised and placed in 20 mM EDTA for 1 h at 37 °C, allowing isolation of the epidermis as a single sheet. Epidermal sheets were rinsed in cold 0.1 M PBS for 5 min, frozen on dry ice, and stored at −80 °C. Total RNA was extracted in TRIzol (Invitrogen) and concentrations measured using a NanoDrop ND-1000 spectrophotometer (ThermoScientific). Complementary DNA was generated using 1 μg of mRNA and SuperScript II Reverse Transcriptase (Invitrogen) in an MJ Mini Personal Thermal Cycler (BioRad). cDNA was amplified using iQ SYBR Green Supermix (BioRad) and an iCycler iQ Multicolor Real-Time PCR detection system (BioRad). Cycle number was recorded with the iCycler program (BioRad). Primer sets were: nerve growth factor (NGF); sense: TTTAAGAAACGGAGACTC and anti-sense: CTGTTGAAAGGGATTGTA; brain derived neurotrophic factor (BDNF; sense: AGAGTGATGACCATCCTT and anti-sense: TGGACGTTTACTTCTTTCA; glial cell line derived neurotrophic factor (GDNF); sense: TTAACTGCCATACACTTA and anti-sense: CTACTTTGTCACTTGTTAG. For normalization, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used: sense: CTCTACCCACGGCAAGTTC and anti-sense: CTCAGCACCAGCATCACC (Integrated DNA Technologies, Coralville, IA).

2.6. Behavioral testing

Thermal and mechanical sensitivity were measured 1 d prior to and 1 and 13 d after treatment was initiated. For thermal testing, mice were allowed to acclimate on a glass surface for 30 min and a radiant heat source (PAW Thermal Stimulator [UCSD], 4.3A) applied to the hind paw plantar surface and time required for the animal to withdraw its paw measured [3]. Each animal was tested 3 times on each hind paw with 5–10 min between tests.