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Letter to the Editor

In vivo study of targeted nanomedicine delivery into Langerhans cells by multiphoton laser scanning microscopy

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Abstract: Epidermal Langerhans cells (LCs) function as professional antigen-presenting cells of the skin. We investigated the LC-targeting properties of a special mannose–moiety-coated pathogen-like synthetic nanomedicine DermaVir (DV), which is capable to express antigens to induce immune responses and kill HIV-infected cells. Our aim was to use multiphoton laser microscopy (MLM) *in vivo* in order to visualize the uptake of Alexa-labelled DV (AF546-DV) by LCs. Knock-in mice expressing enhanced green fluorescent protein (eGFP) under the control of the langerin gene (CD207) were used to visualize LCs. After 1 h, AF546-DV penetrated the epidermis and entered the eGFP-LCs. The AF546-DV signal was equally distributed inside the LCs. After 9 h, we observed AF546-DV signal accumulation that occurred mainly at the cell body. We demonstrated in live animals that LCs picked up and accumulated the nanoparticles in the cell body.

Key words: eGFP-Langerin knock-in mice – *in vivo* – Langerhans cells – multiphoton laser microscopy – nanomedicine formulation

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Background

Near-infrared femtosecond laser-based fluorescence microscopy has rapidly evolved and has become capable to monitor fine morphological changes in Langerhans cells (LCs) (1,2). To track interactions between LCs and DermaVir *in vivo*, knock-in mice expressing enhanced green fluorescent protein (eGFP) under the control of the langerin (CD207) gene were used (3). DermaVir is an HIV therapeutic vaccine candidate to boost HIV-specific T-cell responses (4).

Questions addressed

1. After administering DV with the DermaPrep medical device – that interrupts *stratum corneum* – LCs take up DV via receptor-mediated endocytosis (4). Is DV able to penetrate through the intact skin of a murine ear?
2. DV-loaded LCs migrate into the lymph nodes where they express HIV antigens and induce cytotoxic T-lymphocytes (CTLs) (5,6). Does the intracellular distribution of DV nanoparticles change following their uptake by LCs? Do LCs undergo morphologic changes as a sign of their activation?

Experimental design

AF546-DV was prepared by covalently binding Alexa-546 succinimidyl ester (Life Technologies, Carlsbad, CA, USA) to the amine moieties of polyethyleneimine-mannose (PEIm). DermaVir nanoparticles were prepared using the labelled PEIm and plasmid DNA in triethanolamine buffer (pH 7.6) containing mannitol, as described previously (7). Studies using Langerin-EGFP-DTR-knock-in mice (3) and BALB/c mice were approved by the Semmelweis University Animal Care and Use Committee. The anesthetized animals were laid on a 37°C heated microscope table to keep their body temperature constant. In our experiments, the ears of the AF546-DV-treated animals were examined (after 1, 9 and 24 h of the treatment). The *stratum corneum* interruption was performed with DermaPrep medical device using the exfoliating sponge and stripping tapes to remove residual cell matter from the skin surface. As laser light source, a broadly tunable, femtosecond pulse Ti-sapphire laser (FemtoRose 100TUN NoTouch, R&D Ultrafast Lasers Ltd, Budapest, Hungary) was used that generated approximately 190 fs pulses at a repetition rate of approximately 76 MHz. The laser's central wavelength was set to approximately 890 nm, which assured high two-photon excitation efficiency for both the AF546-DV and eGFP-labelled LCs as well as low background autofluorescence signal originating from the intrinsic autofluorescence of inherent endogenous chromophores of the skin like keratin, NAD(P)H and melanin (8).

Results

AF546-DV penetration into the deep layers of mouse ear skin

We examined the potential of AF546-DV to penetrate through the intact mouse skin barrier, when the natural surveillance activity of LCs focuses on AF546-DV exclusively. Anesthetized Langerin-EGFP-DTR-knock-in mice were placed on the microscope stage, and series of *Z*-stack images were recorded at the place of the treatment at three different time points. The compiled *Z*-stack images are presented in Fig. 1 as composite orthogonal cuts. After 1 h of treatment, AF546-DV penetrated into the epidermis reaching an average depth of approximately 10 μm from *stratum corneum*. After 9 and 24 h, diffuse distribution of AF546-DV was observed in a 5–30 μm depth range (Fig. 1).

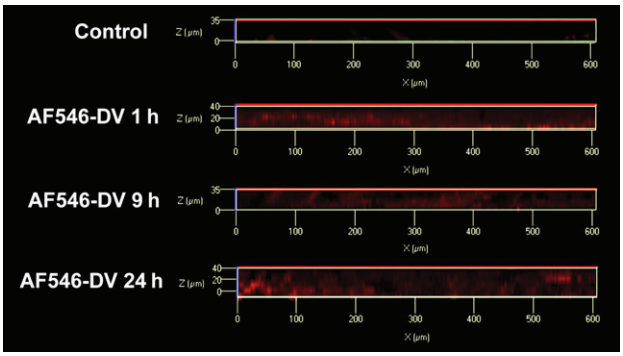

Figure 1. Penetration kinetics of AF546-DV through the stratum corneum in enhanced green fluorescent protein (eGFP)-Langerin knock-in mouse ear *in vivo*. *xz*-Multitracking sections were composed from a stack of *xy*-optical sections with 5 μm distances between the sections. The sections were recorded from the stratum corneum (Z = 0 μm) to the epidermis (Z = 35–40 μm). These representations reveal the penetration profiles of AF546-DV into eGFP-Langerin knock-in mouse skin reaching an average of 20 μm penetration depth underneath the honeycomb-shaped corneocyte layer after 1 h of topical treatment. AF546-DV diffused in the whole depth of the skin after 9 or 24 h despite of the fact that a part of the AF546-DV formula dried on the stratum corneum. Control: intact skin without AF546-DV.

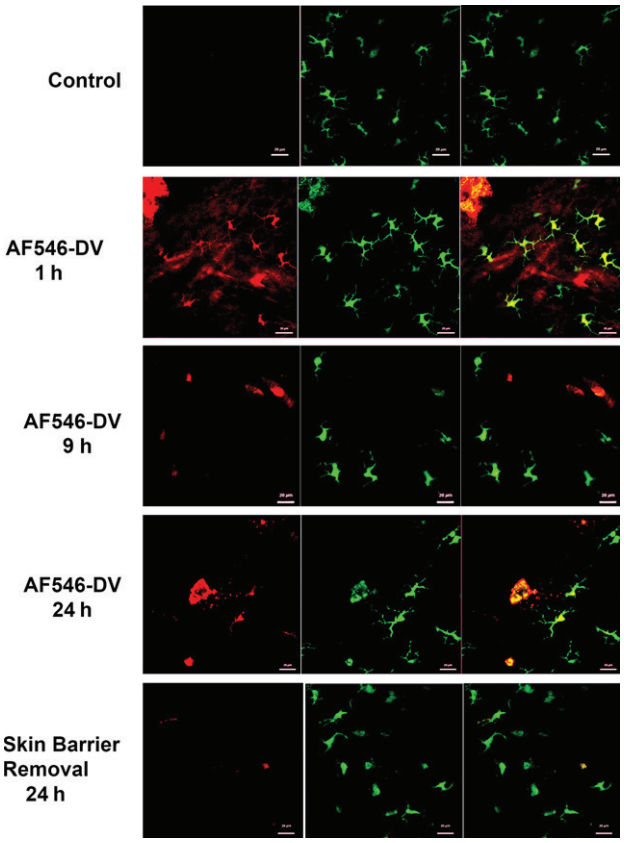

Figure 2. Kinetics of AF546-DV uptake by Langerhans cells (LCs) in eGFP-Langerin knock-in mouse ear *in vivo*. Nearly all LCs had incorporated AF546-DV after 1 h of topical treatment: strong colocalization was detected in both channels [NDD 2 – green/eGFP (middle column) versus NDD 1 – red/AF546-DV (left column)] as presented on the merged pictures (right column). Images of red light emission also revealed that the nanoparticles were distributed homogenously in all parts of the LCs. After 9 h, the intensity of red light emission by AF546-DV decreased significantly and disappeared from the dendrites and concentrated around the nucleus. Intriguingly, after 24 h, the nuclear location as well as a weak signal of AF546 in the dendrites could still be observed. The removal of the stratum corneum resulted in the activation of the vast majority of the LCs characterized by a rounded potato-like shape. The scale bar represents 20 μm.

Uptake kinetics of AF546-DV by LCs

Besides studying the penetration of AF546-DV and its interaction with LCs in the skin, our further aim was to characterize the intracellular route of nanoparticles inside the LCs. In control skin images (no AF546-DV treatment), there was no signal in the red detection channel, NDD2 (565–610 nm) originating from LCs (Fig. 2, left column). After 1 h of topical administration, AF546-DV was distributed nearly homogenously in the epidermis, and specific accumulations were also found at the locations of LCs. This observation was verified by the exact colocalization of the intense AF546-DV (red) signal and the eGFP (green) fluorescence of LCs (Fig. 2 1 h, Figure S1). Nearly all LCs took up the AF546-DV nanoparticles which were distributed homogenously inside the LCs. Morphological changes of the LCs were not observed. These data suggest that AF546-DV was endocytosed by LCs after 1 h. The extended homogeneous AF546-DV signal that was observed after 1 h disappeared after 9 h (Fig. 2 9 h; Figure S1). The AF546-DV signal was observed only as spots that were colocalized with eGFP fluorescence, suggesting that the AF546-DV redistributed in the LCs. The AF546-DV signal disappeared from dendrites and concentrated in the cell body of LCs at the nuclear region (Fig. 2 9 h; Figure S1). After 24 h, AF546-DV was located mainly in the cell body of LCs, too (Fig. 2 24 h; Figure S1). Characteristic morphological marks of activation – potato-like shape of LCs – during AF546-DV administration to the intact skin could not be observed at either time as it was observed in the case of skin preparation with DermaPrep (Fig. 2 Skin Barrier Removal). All these data suggest that AF546-DV was successfully taken up by majority of LCs and after several hours, it concentrated inside the cell body, at nuclear region.

Conclusions

Transcutaneous immunization (TCI) targeting the LCs of the skin has received much attention due to its safe, needle-free and non-invasive antigen delivery. Third-generation TCI technologies include the disruption of the *stratum corneum* in a highly localized manner (9). Materials that self-assemble with nucleic acids into nanocomplexes are widely used in biological and biomedical investigations (10), like in the clinical application of DermaVir and DermaPrep (5). In our experiments, natural surveillance of LCs focused mainly on their interaction with AF546-DV because hairs and *stratum corneum* remained intact, in contrary to clinical application of DermaVir. AF546-DV penetrated the relatively thin intact skin of the ear and saturated the epidermis in 1 h. The AF546-DV was taken up by LCs specifically. After 9 h of topical administration, the surplus of AF546-DV was drained off from the epidermis reflecting lymphatic drainage of the unbound probes. These results suggest that AF546-DV efficiently penetrates the thin dermal layers of ear. The AF546-DV uptake by LCs was observed in the epidermis at each of the examined times (Fig. 2). It was also found that AF546-DV concentrated at the cell body of LCs. It suggests a specific intracellular transportation of AF546-DV in accordance with the intrinsic behaviour of PEIm and DermaVir formula (7,11). However, AF546-DV administration to the intact

skin did not induce morphological changes in the LCs as sign of their activation, unlike the skin preparation with DermaPrep. The developed optical set-up of multiphoton laser microscopy allowed monitoring the penetration of AF546-DV and its interaction with LCs *in vivo* (12).

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Author contributions

AK, ZC and RS optimized the fluorescent labelling and optical excitation and detection system for low background and minimum cross-talk measurements; ZC, DH, AK and RS performed the multiphoton laser microscopy measurements *in vivo*; AK, ZC, OL and ET evaluated the data; AK, ET and RS wrote the paper.

Conflict of interest

AK and RS hold shares in R&D Ultrafast Lasers Ltd. ZC, ET and OL hold share in Genetic Immunity Inc. The other author declares no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *In vivo* intracellular localization of AF-546-DV in LCs of eGFP-Langerin knock-in mouse ear.