Characterization of Nanoparticle Uptake by Epidermal Langerhans Cells in eGFP-Langerin Knock-in Mice by Multiphoton Laser Scanning Microscopy *in vivo*

A. Kolonics[1,2], Zs. Csiszovszki[3], E. Tőke[3], O. Lőrincz[3], P. Stoizner[4], N. Romani[4], B. Malissen[5], J. Lisziewicz[3], *R. Szipőcs[1,2]

[1]*Institute for Solid State Physics and Optics of Wigner RCP, P.O. Box 49, H-1525 Budapest, Hungary*
[2]*R&D Ultrafast Lasers Ltd, P.O. Box 622, H-1539 Budapest, Hungary*
[3]*Genetic Immunity, Berlini utca 47-49, H-1045 Budapest, Hungary*
[4]*Universitätsklinik für Dermatologie & Venerologie Abteilung für Experimentelle Dermatologie, Labor für Langerhanszellforschung (4-U1-015) Anichstrasse 35, A-6020 Innsbruck, Austria*
[5] *CIML Pare Scientifique de Luminy Case 906 13288 Marseille Cedex 9, France*

**corresponding author*: szipocs.robert@wigner.mta.hu*

Abstract: Fluorescently labelled polyethylenimine-mannose/DNA nanoparticles caused morphological and intracellular changes in Langerhans cells as studied by multiphoton laser microscopy *in vivo* in ear of knock-in mice expressing enhanced Green Fluorescent Protein under control of langerin gene.

OCIS codes: (180.4315) Nonlinear microscopy; (170.1870) Dermatology

1. Introduction

Epidermal Langerhans cells (LCs) represent a prototypic immature dendritic cell (DC) subset that is characterized by the localization within the outermost layer of the skin and the extension of long dendritic processes [1-2]. A major task of LCs in the steady state is to capture surrounding materials (including antigens) by macropinocytosis, endocytosis, and phagocytosis. Upon sensing inflammatory signals, however, LCs exhibit rapid and profound changes in morphology, gene expression profile, surface phenotype, and function. To track interactions of LCs *in vivo*, knock-in mice expressing enhanced green fluorescent protein (eGFP) under the control of the langerin (CD207) gene were developed [3]. High resolution morphological details manifesting three-dimensional (3D) organization of LCs and their associated intracellular structures within the skin tissue are often difficult to obtain using routine histology and conventional fluorescence [4]. The depth through which confocal LSM can obtain high resolution 3D images is rather limited because of the low penetration depth of ultraviolet (UV) and visible (VIS) lasers used. In the last 20 years, near infrared (NIR) femtosecond laser-based fluorescence microscopy has rapidly evolved and became capable to monitor fine morphological changes in Langerhans cells [5]. These developments paved the way to be able to answer several key questions regarding transdermal routes of different nanomedicines. The encapsulation of active substances is a common pharmaceutical strategy to modify the transport and release properties of a drug. Especially to nanoparticulate systems, great potential is attributed in the field of drug delivery. This is partly due to the fact that sensitive drugs can be hidden from degradation in the particles [7]. Functional coatings of the particles may allow the targeted accumulation and release of drugs at their therapeutic sites [8-9]. Nanoscale polymeric drug vehicles have also been proposed for transdermal delivery [10]. Polyethylenimine (PEI)-mediated transient gene expression processes are expected to play an increasingly important role in the development of protein therapeutics within the scope of biopharmaceutical applications [11]. In the presence of PEIs, DNA molecules are condensed and compacted into PEI/DNA complex particulates, acquiring the capability to interact with electrostatically negative moieties such as heparin sulfate proteoglycans on the surfaces of cells for endocytosis/phagocytosis [12]. Genetic Immunity have been developing DermaVir, an HIV therapeutic vaccine product candidate, to decelerate HIV disease progression by boosting HIV-specific T cell responses. The clinical DermaVir product contains a single plasmid DNA immunogen formulated with a cationic polymer linear mannosylated polyethylenimine (PEIm) to form a pathogen-like synthetic nanoparticle of about 200-300 nm that supports gene expression and antigen presentation and gene expression in dendritic cells [13]. After topical administration with the DermaPrep delivery medical device, DermaVir is taken up via receptor-mediated endocytosis by epidermal Langerhans cells and targeted to the lymph node dendritic cells where it expresses the pDNA encoded HIV antigens and induces precursor/memory T cells with high proliferation capacity [14]. Positive clinical results on safety, immunogenicity and viral load reduction have been observed following DermaVir treatment in HIV-infected individuals [15].
In this study, we describe the novel application of a NIR two-photon LSM used in combination with advanced 3D image analysis and quantification software to examine the kinetics of uptake of DermaVir by LCs and to further characterize cellular fate of the nanoparticles.

2. Experimental setup

A broadly tunable, femtosecond pulse Ti-sapphire laser (*FemtoRose 100TUN NoTouch*, product of R&D Ultrafast Lasers Ltd [16]) generates nearly transform limited, τ_{FWHM} ~190 fs pulses at a repetition rate of ~76 MHz for our 3D microscopy measurements. The laser utilizes ultra broadband chirped mirror (UCBM) technology for broad tuneability (690 nm to 1050 nm) without changing the cavity optics [17]. The laser central wavelength is set to ~890 nm, which assures high two-photon excitation

efficiency for both the Alexa-546 dye-labeled Dermavir nanoparticles and the EGFP-labeled LC-s, but low background autofluorescence signal from the intrinsic autofluorescent molecules in the skin, such as keratin, NAD(P)H, melanine [18]. Note, that the SHG signal from the collagene network is generated at around 445 nm, which is out of the transmission band of our bandpass filters (see later). For 3D imaging, we used a two-channel, Axio Examiner LSM 7MP microscope (Carl Zeiss, Germany). Scanning in the x–y plane was achieved with galvanometer mirrors that provided up to video-rate image acquisition. Subfemtolitre excitation beam focusing was achieved using a high-numerical-aperture (N.A.~1.3) 40x water immersion objective (Zeiss), with a working distance of about 1 mm. Stepper motor control of the objective lens focus enabled scanning along the optical z-axis with a minimum step size of 150 nm. The average power of the laser beam reaching the sample is typically set to ~ 10-15 mW by a built-in acousto-optic device, which assures thermal-damage free excitation in our *in vivo* measurements (pixel dwell time is ~ 6.3 μs). For cross-talk free recording of the flourescence signal from the EGFP and Alexa-546 dye, two properly chosen bandpass filters (BPF) and a corresponding dichroic filter was placed in front of the the non-descanned (NDD) detectors: a BPF1 (green) BPF2 (red) filters having transmission bands in the 500-550 nm and the 565-610 nm wavelength regimes, respectively. Alexa546-DermaVir was prepared by covalently bounding Alexa-546 succinimidyl ester (Invitrogen) to the amine of PEIm according manufacturer's procedure. Unbonded dye was extensively washed out, the labeling efficiency was measured by UV/VIS spectroscopy. DermaVir nanoparticles were prepared using the labeled PEIm and a plasmid DNA in triethanolamine buffer (pH 7.6) containing mannitol conform the standardized mixing procedure of Genetic Immunity [13]. The formed nanoparticles were quality controlled according the clinical DermaVir specification. Topical administration of Alexa546-DermaVir to the mouse ear was performed using the DermaPrep administration method apart from removing *stratum corneum* [19].

3. In vivo experiments

In vivo NIR two-photon LSM was used to image the fluorescence signals of both Alexa546-DermaVir and eGFP-Langerin cells in mouse ears at different time points after topical application of DermaVir. Our findings provide evidence that DermaVir penetrates through murine epidermis and it concentrates around LCs in the epidermis. The Alexa546-DermaVir was endocytosed by LCs as early as 1 hour after treatment and showed homogenous distribution in the LCs (Figure 1.a). After 9 hour the fluorescence of DermaVir was mainly observed intracellularly, close to nucleus in LC suggesting successful uptake of DermaVir into LC (Figure 1.b). At the 24h time point, DermaVir showed either unaltered localization and in some cells the vaccine was located close to cell surface in activated LC which shows a round cell body. In addition slow decay of the signal was also observed around the nucleus. (Figure 1).

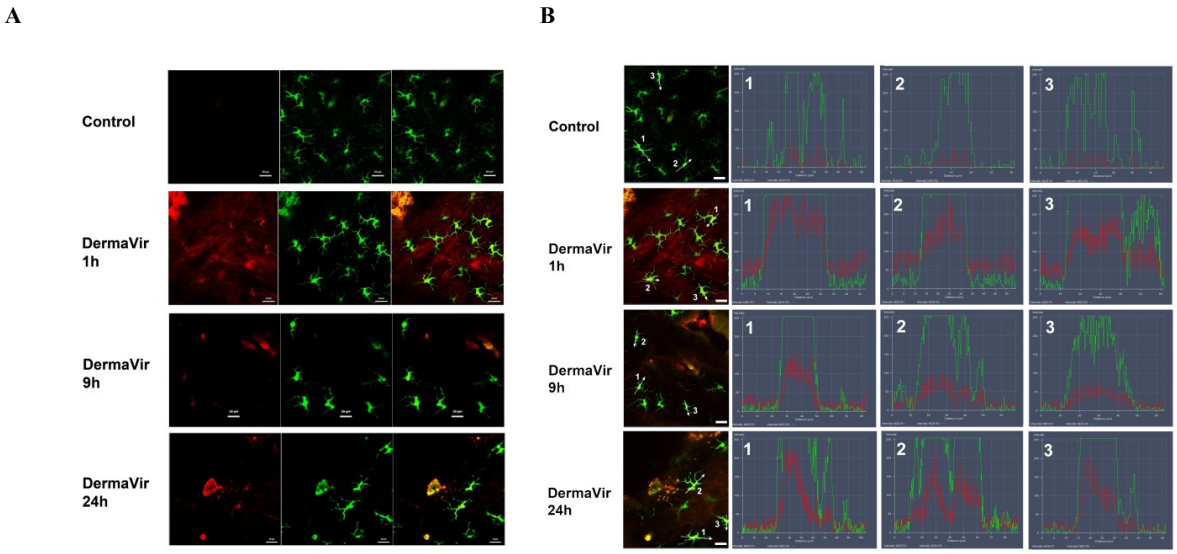

Fig.1. Uptake kinetics of DermaVir-Alexa546 by LCs in eGFP-Langerin knock-in mouse ear *in vivo* A, Cellular uptake of Alexa546-DermaVir by Langerhans cells. After 1 hour of topical treatment by Alexa-546-DermaVir colocalisation was detected between both channels (NDD R2 – green/eGFP vs NDD R1 – red/ Alexa-546-DermaVir) suggesting that the nanoparticles were taken up by LCs. Image of red light emission also reveals that the nanoparticles are homogenously distributed in all part of the LCs. Alexa-546-DermaVir staining was found mainly around the nucleus of the cells after 9 and 24 hours of the treatment too. (Scale bar represents 20 μm) **B,** Intracellular localization of Alexa-546-DermaVir in LCs of eGFP-Langerin knock-in mouse ear *in vivo* In control cells marginal red signal is detected from over-excitation of eGFP-Langerin as it was shown by line analysis of 3 independent cross section of cells. After 1 hour of Alexa-546-DermaVir treatment strong red signal is detected in dendrites and cell body (#3, 40-60 μm). After 9 hours of the treatment the red signal is somehow lower and mainly originated from the cell nucleus (#2, #3). Similar results were found after 24 hours of the treatment, however the red signals were more intense and concentrated (#1 & #3).

4. Conclusion

We could demonstrate that the synthetic pathogene-like nanoparticle, DermaVir was up taken by LCs in 1 hour and DermaVir or its degraded parts concentrated around nucleus suggesting specific intracellular transportation. The decrease of Alexa-546-DermaVir fluorescence around the nucleus after 9 and 24 hours may suggest that DermaVir started to degrade and inherent plasmid DNA are released into the nucleus of the Langerhans cells as expected. The established detection system is exploitable to characterize efficiency of nanomedicine formulation in either immuno-dermatological response and in specific intracellular changes. In addition it can be also useful in model situations like virus disease progression, vaccination, ultraviolet and other radiation exposure, as well as unraveling allergic character of different topically applied drugs.

5. References

1. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245–52
2. Romani N, Holzmann S, Tripp CH, Koch F, Stoitzner P (2003) Langerhans cells – dendritic cells of the epidermis. Apmis 111:725–40
3. Kissenpfennig A, Henri S, Dubois B, Laplace-Builhé C, Perrin P, Romani N, Tripp CH, Douillard P, Leserman L, Kaiserlian D, Saeland S, Davoust J, Malissen B. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity. 2005 May;22(5):643-54.
4. Ito H, Takekoshi T, Miyauchi M, Ogawa I, Takata T, Nikai H, Takemoto K. 1998. Three-dimensional appearance of Langerhans cells in human gingival epithelium as revealed by confocal laser scanning microscopy. Arch Oral Biol 43:741–744.
5. Tirlapur UK, Mulholland WJ, Bellhouse BJ, Kendall M, Cornhill JF, Cui Z. Femtosecond two-photon high-resolution 3D imaging, spatial-volume rendering and microspectral characterization of immunolocalized MHC-II and mLangerin/CD207 antigens in the mouse epidermis. Microsc Res Tech. 2006 Oct;69(10):767-75.
6. Frugé RE, Krout C, Lu R, Matsushima H, Takashima A. Real-time visualization of macromolecule uptake by epidermal Langerhans cells in living animals. J Invest Dermatol. 2012 Mar;132(3 Pt 1):609-14.
7. Volodkin DV, Sukhorukov GB, Larionova NI (2004) Protein encapsulation via porous CaCO3 microparticles templating. Biomacromolecules 5: 1962–72
8. Dinauer N, Von Briesen H, Balthasar S, Weber C, Kreuter J, Langer K (2005) Selective targeting of antibody-conjugated nanoparticles to leukemic cells and primary T-lymphocytes. Biomaterials 26:5898–906
9. Kotrotsiou O, Kotti K, Dini E, Kammona O, Kiparissides C (2005) Nanostructured materials for selective recognition and targeted drug delivery. J Phys Conf Ser 10:281–4
10. Alvarez-Roman R, Naik A, Kalia YN, Guy RH, Fessi H (2004c) Enhancement of topical delivery from biodegradable nanoparticles. Pharmaceut Res 21:1818–25
11. Baldi L, Hacker DL, Adam M, Wurm FM (2007) Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol Lett 29:677–684
12. Godbey WT, Wu KK, Mikos AG (1999) Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci USA 96:5177–5181
13. Tőke ER, Lőrincz O, Somogyi E, Lisziewicz J. Rational development of a stable liquid formulation for nanomedicine products. International Journal of Pharmaceutics 2010;392(1–2):261–7.
14. Lisziewicz J, Trocio J, Whitman L, Varga G, Xu JQ, Bakare N, et al. DermaVir: a novel topical vaccine for HIV/AIDS. Journal of Investigative Dermatology 2005;124(1):160–9.
15. Gudics A, Lisziewicz J. Nanomedicine for dendritic cell-targeted immunotherapy. European Journal of Nanomedicine 2010;3(1):25–9, http://clinicaltrials.gov/ Phase I: NCT00712530; Phase II: NCT00270205, NCT00918840, NCT00711230
16. www.fslasers.com/products
17. E. J. Mayer, J. Möbius, A. Euteneuer, W. W. Rühle, and R. Szipőcs, "Ultrabroadband chirped mirrors for femtosecond lasers," Opt. Lett. **22**, 528 (1997)
18. Breunig, H.G., H. Studier, and K. König, Multiphoton excitation characteristics of cellular fluorophores of human skin in vivo. Opt Express, 2010. 18(8): p. 7857-71
19. www.geneticimmunity.com/IFU