The role of C-type lectin receptors in human skin immunity: immunological interactions between dendritic cells, Langerhans cells and keratinocytes
van den Berg, L.M.

Citation for published version (APA):
van den Berg, L. M. (2013). The role of C-type lectin receptors in human skin immunity: immunological interactions between dendritic cells, Langerhans cells and keratinocytes
CHAPTER 6

HYALURONIDASES REGULATE DYNAMIC CELLULAR INTERACTIONS AND MIGRATION OF HUMAN LANGERHANS CELLS

Linda M. van den Berg
Esther M. Zijlstra-Willems
Teunis B.H. Geijtenbeek

Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
ABSTRACT

Epidermal Langerhans cells (LCs) express the C-type lectin receptor langerin that is an adhesion receptor for hyaluronic acid (HA). HA is expressed by epidermal keratinocytes (KCs) and here we investigated whether langerin mediates interactions with KCs and how these interactions are regulated. LCs interacted with KCs through langerin-HA. Notably, loss of HA on KCs induced LC migration in an ex vivo skin culture model, suggesting that langerin-HA interactions control LC localization and migration. Maturation of LCs, led to upregulation of hyaluronidase-1 (hyal-1) and hyal-2, which cleaved HA and enabled LC migration. Hyaluronidase upregulation is a general mechanism to regulate langerin interactions since hyal-2 upregulation in dendritic cells (DCs) after LC-DC clustering cleaved HA from the DC surface and decreased LC-DC interaction. Thus, our data show that langerin-HA interaction controls the localization of LCs, and that hyaluronidases regulate dynamic cellular interactions. Vaccination strategies inducing hyaluronidase expression in epidermis could facilitate LC maturation and migration and might enhance immunogenicity.

INTRODUCTION

Langerhans cells (LCs) are professional antigen presenting cells residing in close proximity of keratinocytes (KCs) in human epidermis. LCs act as sentinels of the immune system and sample the surrounding for intruding pathogens, which are captured and subsequently, antigens are presented by LCs to T cells in the lymph nodes. In the epidermis, LCs interact with KCs via E-cadherin/E-cadherin adherence junctions. However, the homophilic E-cadherin interaction between LCs and KCs turned out to be dispensable for LC maintenance in the epidermis. Therefore, we studied the mechanisms by which LCs interact with other cell types in epidermis and dermis, and how LC migration is regulated.

Human LCs exclusively express the C-type lectin receptor (CLR) langerin, which functions as pathogen recognition receptor (PRR) by recognizing pathogenic saccharides on viruses, fungi and bacteria, such as mannose, N-acetyl-glucosamine (GlcNAc), fucose and beta-glucans. Recently, we showed that langerin interacts with the glycosaminoglycan hyaluronic acid (HA) on DCs. In addition, HA is also expressed by other cells including KCs, which suggest that LCs might be able to interact via langerin with different cell types.

KCs produce HA via HA synthases and subsequently extrude the polymer via a transporter into the extracellular matrix. HA metabolism is regulated by hyaluronidase enzymes in tissues and skin. HA high molecular weight (HA HMW) (> 1*10^6 kDa) is digested into low molecular weight (LMW 20-40 kDa) by hyaluronidase-2 (hyal-2), which is glycosylphosphatidylinositol-anchored to the cell membrane. Subsequently, HA is endocytosed and intracellular processed by hyal-1. Hyal-1 is also found in serum and urine and is larger than intracellular hyal-1 (57 kDa and 45 kDa, respectively). Knocking out hyal-2 is lethal for mice highlighting the importance
of HA and HA metabolism for systemic homeostasis.

Since HA is abundantly expressed in epidermis and dermis, the process of langerin-HA binding should be highly regulated. Here we showed that LCs upregulate both hyal-1 and -2 that digest HA and release LCs from the environment. Upon LC-DC clustering, DCs mature and upregulate hyal-2, which subsequently degraded HA from the DC cell surface and decreased clustering. Thus, HA expression and cellular LC interactions via langerin are highly regulated by the expression of hyal-1 and hyal-2. Control of HA by hyaluronidases regulates LC migration and cellular interactions, and therefore, manipulation of these mechanisms might provide novel strategies to regulate immune activation.

**RESULTS**

**Langerin binding pattern in human skin follows HA expression**

Here we investigated the interaction of LCs with KCs in human epidermis. LCs resided in close proximity of KCs (Fig 1a). Keratin+ KCs expressed HA (Fig 1a, b), which is a ligand for langerin. Besides epidermis, we observed HA positive cells in the dermis (Fig 1c), which were identified as DCs (data not shown, chapter 5). Next we investigated whether langerin interacts with HA expressed in epidermis and dermis using FITCylated soluble langerin. The pattern of soluble langerin binding in skin was the same as the expression pattern of HA (Fig 1c, d) and we observed binding of langerin to KCs in the epidermis and to CD11c+ HA+ DCs in dermis (Fig 1d). These data strongly suggest that HA expressed by KCs is a ligand for langerin.

![Figure 1: Langerhans cells reside with keratinocytes in epidermis](image-url)

Skin sections were snap frozen and cryo sections were stained for pankeratin and CD1a (A) and for HA (B,C). Soluble langerin binding in skin was determined by incubating cryo sections with soluble Langerin-FITC (D). Sections were counterstained with CD11c to identify DCs. Sections were subsequently analyzed by confocal scanning laser microscopy; bars represent 50 µm.
Langerin binds to HA on KCs

Primary human KCs were isolated from human skin and we investigated the interaction with langerin. KCs were incubated with unlabeled soluble langerin, and binding was detected by anti-langerin antibodies. Soluble langerin bound efficiently to KCs, which was blocked by mannan and blocking antibody to langerin (10E2) (Fig 2a). KCs expressed HA on the cell surface, which could be removed by treatment of KCs by the enzyme hyaluronidase (Fig 2c). Langerin binds to HA expressed by KCs, since removal of HA by hyaluronidase diminished langerin binding (Fig 2b). To investigate the role of hyaluronidases in LC migration, human epidermis was floated onto PBS supplemented with or without hyaluronidase for 5 hours. We observed enhanced LCs migration out of epidermis in the presence of hyaluronidase (Fig 2d) whereas no KCs were observed in the supernatant (data not shown). These data show that enzymatic degradation of HA on KCs facilitates LC migration out of the epidermis, strongly suggesting HA-langerin interaction is retaining LCs in epidermis and loss of the interaction induces migration.

Figure 2: Langerin interacts with HA on KCs

Soluble langerin was incubated with KCs and binding was determined with an anti-langerin antibody by flow cytometry. Langerin specificity was determined by blocking langerin antibody 10E2 and mannan; graph is representative for 3 donors (A). KCs were control-treated or hyaluronidase treated at pH5.2 and langerin binding was determined by flow cytometry; graph is representative for 3 donors (B). HA CD44Fc coupled to fluorescent beads was used in a beads-binding assay to determine the HA expression level at the KC cell surface; mean and SD of 3 donors; experiments in duplicates (C). Epidermal sheets of one square centimetre were floated onto PBS pH5.2 supplemented with or without hyaluronidase. LC migration was determined as absolute number of migrating cells by flow cytometry; mean and SD of 3 donors; experiments in hexaplicates (D). Paired student's t-test; p<0.01.
LCs upregulate hyal-1 and hyal-2 for migration out of epidermis
We investigated how the interaction between LCs and KCs is regulated upon migration. We analyzed the expression of hyal-1 and hyal-2 in LCs and KCs. We mimicked infection with pathogens by stimulating KCs with TNF or Poly(I:C), ligands for TNF receptors and TLR3 respectively. KCs responded to these triggers by the production of pro-inflammatory cytokine interleukin-8 (IL-8; data not shown). However, KCs did neither upregulate expression of hyal-1 nor hyal-2 upon stimulation (Fig 3a), and HA expression levels on KCs remained stable upon Poly(I:C) and TNF stimulation (Fig 3b).

Figure 3: Upregulation of hyal-1 and hyal-2 in mature LCs. Keratinocytes were stimulated with TNF and poly(I:C) and were immuno-blotted for hyal-1 and hyal-2; graph is representative for 2 donors (A). CD44Fc coupled to fluorescent beads was used in a beads-binding assay to determine the HA expression level at the KC cell surface after TNF and poly(I:C) stimulation; mean and SD of 3 donors in duplicates (B). Immature and mature LCs were isolated from the same donor. Hyal-1 and hyal-2 expression was determined by immuno-blotting cell lysates; graph is representative for 3 donors (C). Mature LCs were fixed and stained for langerin and hyal-2 and were analyzed by confocal scanning laser microscopy; pictures are representative for 3 donors; bars represent 10 µm (D). Langerin was immuno-precipitated from mature LC lysates and immuno-blotted for langerin and hyal-2; representative for 2 donors (E).
These data suggest that KC activation by itself does not downregulate HA to facilitate LC migration out of epidermis. Next we prepared immature LCs and mature migratory LCs from the same donor to compare hyaluronidase expression levels. In general, immature LCs are MHC class II intermediate and CD86 negative, whereas mature LCs express high MHC class II and CD86 (data not shown). Compared to immature LCs, mature LCs have increased levels of both hyal-1 and hyal-2 (Fig 3c) suggesting that maturation increased hyal-1 and -2 levels. Hyal-2 upregulation might cleave cell surface HA from neighbouring KCs, which is subsequently internalised and degraded by hyal-1. Next, we investigated the localization of hyal-2 in mature LCs by confocal scanning laser microscopy. Hyal-2 was present at the cell surface of mature LCs. At the surface, langerin and hyal-2 partly colocalized (Fig 3d). Indeed, co-immuno-precipitation showed that hyal-2 co-immuno-precipitated with langerin (Fig 3e), which confirms langerin and hyal-2 colocalization observed in the cell membrane (Fig 3d). These data strongly suggest that hyal-2 cleaves HA to liberate LCs for migration toward the dermis.

**DCs downregulate HA upon maturation**

Besides interactions with KCs, LCs also cluster with DCs for antigen transfer and induction of immune responses, as described in chapter 5. Although mature LCs express hyal-2, they strongly clustered with DCs in vitro (Fig 4B). Immature DCs expressed high levels of HA (Fig 4a), which was downregulated after maturation with TLR4 and -3 ligands LPS and Poly(I:C), respectively (Fig 4a). LC-DC interaction was followed in time and was markedly reduced upon DC maturation strongly suggesting downregulation of HA on DCs disables LC-DC clustering (Fig 4b). Therefore, we investigated the expression of hyaluronidases by DCs. DCs constitutively expressed hyal-1 (Fig 4c), which might degrade short HA that have been generated by hyal-2. Hyal-1 expression levels were independent of the maturation status of DCs (Fig 4c). Notably, hyal-2 protein levels rapidly increased upon DC maturation (Fig 4c). Hyal-2 was measured at different time points and after 16 hours hyal-2 levels started to increase, which further increased up to 48 hours (Fig 4c, lower part). To investigate whether hyal-2 in DCs is responsible for downregulation of extracellular HA, hyal-2 mRNA was silenced with RNAi in DCs (supplementary figure 1). HA expression decreased after DC maturation with LPS (Fig 4d) but hyal-2 silencing completely rescued the expression of HA (Fig 4d). Next, we investigated soluble langerin binding to DCs. Langerin binding to LPS-matured DCs decreased, whereas silencing of hyal-2 resulted in a significant rescue of langerin binding. Therefore our data strongly suggest that maturation of DCs and subsequent upregulation of hyal-2 results in downregulation of HA expression and subsequent loss of LC-DC clustering.

**Mature LCs induce maturation and hyal-2 upregulation in DCs**

We have shown that LCs and DCs transfer antigens during clustering (chapter 5) and that DC maturation abrogates LC-DC clustering (Fig 4B). To investigate whether LCs can induce DC maturation, LCs were co-cultured with DCs and expression of maturation markers was investigated by flow cytometry. Co-culturing of DCs with LCs led to
Figure 4: DCs upregulate hyal-1 to downregulate HA expression

CD44Fc coupled to fluorescent beads was used in a beads-binding assay to determine the HA expression level at the DC cell surface after LPS and poly(I:C) stimulation; graph is representative for at least 5 donors; mean and SD of experiment in duplicates (A). LCs were labelled with hydroethidium and DCs were labelled with CFSE and clustering was monitored in time by flow cytometry (B). DCs were stimulated for different time point with LPS and poly(I:C). Cell lysates were immuno-blotted for hyal-1 and hyal-2; graph is representative for at least 3 donors (C). DCs were silenced for hyal-2 and stimulated with LPS. HA expression was determined using CD44Fc fluorescent beads by flow cytometry analysis; mean and SD of 3 donors (D). DCs were silenced for hyal-2 and stimulated with LPS and soluble langerin binding was determined by flow cytometry; mean and SD of 5 donors; paired student’s t-test; p<0.05 (E).

increased expression of CD86 and partial upregulation of CD80 and HLA-DR to the same levels as LPS-matured DCs (Fig 5a). Although LCs were already activated upon migration, we observed a strong upregulation of CD80, and a slight upregulation of CD86 and HLA-DR by co-culturing LCs with DCs (Fig 5b). These data suggest that the interaction between LCs and DCs is bidirectional and induces maturation of both DC subsets. The DC subsets were isolated after co-culture for 20 hours into two
populations based on DC-SIGN expression and expression of hyal-1 and hyal-2 was analysed. Co-culture of LCs even further upregulated both hyal-1 and hyal-2 expression after interaction with DCs (Fig 5c). In addition, co-culture of DCs with LCs upregulated the hyal-2 protein to the same extent as LPS (Fig 5c). Therefore our data suggest that LCs and DCs regulate their clustering by reciprocal upregulation of hyaluronidases upon maturation. Hyaluronidases downregulated HA expression on DCs, and decreased LC-DC clustering following maturation.

Figure 5: LCs induce maturation of DCs and upregulation of hyal-2, and vice versa
LCs and DCs were co-cultured overnight and analysed for expression of maturation markers CD86, CD80 and HLA-DR by flow cytometry; graphs are representative for 3 donors (A) and 2 donors (B). LCs and DCs were co-cultured overnight and separated by MACS microbeads cell separation based on DC-SIGN expression. Cells were lysed and immuno-blotted for hyal-1 and hyal-2; graph is representative for 2 LC donors with 2 DC donors (C).
DISCUSSION

Langerin is a cellular adhesion receptor that interacted with HA expressed on the surface of both KCs and DCs and here we identified the molecular mechanisms regulating these interactions. Maturation of LCs induced upregulation of both hyal-1 and hyal-2, which decreased HA expression on KCs releasing LCs from the interaction with KCs. Similarly, hyaluronidases control LC-DC interactions. During LC-DC clustering, LCs induced maturation of DCs, upregulation of hyal-2 on DCs. The expression of hyal-2 by DCs induced cleavage of HA and downregulation of HA expression on the DC cell surface, which could be reverted by hyal-2 silencing. In addition, hyal-2 expression downregulated HA on the DC cell surface, which abrogated LC-DC clustering. Thus, cellular interactions of LCs are mediated by HA and controlled by hyaluronidases.

HA is not only expressed by KCs and DCs, but also by fibroblasts, endothelial cells, activated T cells and tumor cells. Langerin could therefore also facilitate interaction between LCs and a broad spectrum of other cell types in the body. Here we described langerin binding to HA on KCs, which could be crucial for retaining the LC network in epidermis. Next to langerin-HA binding, LCs also interact with KCs via homophilic E-cadherin adherence junctions and therefore, it had been assumed that E-cadherin interaction between KCs and LCs regulates adhesion and migration from epidermis. However, a murine DC-Ecad model showed that the homophilic E-cadherin interaction between LCs and KCs is dispensable for LC maintenance in the epidermis. By treating human skin with hyaluronidase, we showed enhanced LC migration from epidermis. Thus, our data suggest that HA retains LCs in the epidermis via the cellular attachment receptor langerin and degradation of HA in the epidermis leads to migration.

HA is highly conserved among vertebrates and also present in the capsule of bacteria, such as streptococci. HA is a unique glycosaminoglycan (GAG), since it is the only major GAG that is not sulphated and the only GAG not attached to a core protein. HA is produced by HA synthases and extruded though the extracellular matrix, where it can reach high molecular weight sizes (HMW) more than 1*10^6 kDa. The turnover rate of HA in skin is between 0.5-3 days. After injury or during inflammation, HA is degraded or broken down into LMW fractions (20-40kDa), which recruit and activate leukocytes via CD44, TLR4 and TLR2. HA LMW might function as danger signal for LCs via langerin or TLR2 increasing maturation and migration. It has been reported that HA plays a role in DC - T cell interactions probably via CD44 interactions. We here showed that HA is involved in LC-KC and LC-DC interactions via the CLR langerin expressed by LCs.

Catabolism of HA in somatic tissues is regulated for the greater part by hyal-1 and hyal-2. Hyal-2 is GPI-anchored to the cell membrane and cleaves HA HMW into LMW fragments (20 kDa). Then, HA fragments are further degraded intracellularly by hyal-1. The importance of HA catabolism is highlighted by the fact that hyal-2 knock out mice are not viable. We showed both hyal-1 and hyal-2 are upregulated in mature LCs. Hyal-2 on LCs supposedly cleaves HA expressed by KCs to enable LC migration and hyal-1 clears the HA LMW fragments from the surrounding,
minimizing local inflammation. For hyal-1 it has been shown that its gene has a transcription factor binding site for NF-κB, Egr-1 and AP-2. NF-κB is activated upon DC maturation via TLR or CLR stimuli and therefore, NF-κB could account for hyal-1 upregulation in mature LCs. Little is known about transcriptional control of hyal-2, however our data suggest that transcription factors involved in LC or DC maturation, such as NF-κB, could also be involved in hyal-2 transcription.

We analyzed expression of hyal-1 and hyal-2 by immuno-blotting. We observed in both LCs and DCs a putative hyal-1 form of 45 kDa, which matches with the predicted size for intracellular hyal-1. For both LCs and DCs we found a hyal-2 band of ~40 kDa, which is slightly smaller than expected (50-52 kDa). This smaller hyal-2 could be specific for LCs and DCs due to alternative splicing or different glycosylation of the protein. Most research on hyal-2 so far focused on hyal-2 (over)expression in cell lines, hyal-2 expression in animal models, or in epithelial human cells and platelets. In human chondrocytes two bands for hyal-2 are observed, ~40kDa and ~55 kDa. We hypothesize hyal-2 detected in LCs and DCs is functional, but differently glycosylated, or even a smaller isoform than the hyal-2 described so far.

We have identified the physiological molecular mechanism for the regulation of interaction between LCs and KCs as well as DCs. These data are relevant for the design of vaccination strategies to improve LC migration. Furthermore, inhibition of hyaluronidases might provide a strategy to prevent over activation by LCs and retain them in skin.

**SUPPLEMENTARY FIGURE**

Supplementary Figure 1: RNAi silences hyal-2 mRNA
DCs were silenced for hyal-1 and were stimulated with LPS. mRNA was isolated from cell lysates and copied to cDNA. Hyal-1 mRNA expression was quantified by real time PCR (A, B, C).

**ACKNOWLEDGEMENTS**

We are grateful to the members of the Host Defense group for their valuable input. We would like to thank the Boerhaave Medical Center (Amsterdam, the Netherlands), Dr. A. Knottenbelt (Flevoclinic, Almere, the Netherlands) and Prof. Dr. C.M.A.M. van der Horst (Academic Medical Center, Amsterdam, the Netherlands) for their valuable support. We would like to thank Dr. M.E. Taylor for providing us with soluble-langerin-FITC. This work was supported by the Dutch Burns Foundation (08.109, LMvdB) and the Dutch Scientific Organization (NWO; VICI 918.10.619, EZW; TBHG).
**MATERIAL AND METHODS**

**Antibodies, proteins and enzymes**
- anti-cytokeratin (AE1/AE3; Dako);
- anti-CD1a (NA1/34; SantaCruz);
- 10E2 (anti-Langerin);
- rabbit-anti-Hyal1, rabbit-anti-Hyal-2 (both Abcam);
- goat-anti-langerin (R&D);
- Goat-anti-Human-Fc-biotin (Jackson);
- anti-CD11c-PE (S-HCL-3; BD);
- DCCGM4-PE (anti-Langerin-PE; Beckman Coulter);
- anti-DC-SIGN-FITC (R&D);
- anti-CD86-PE;
- anti-CD80-PE;
- anti-HLA-DR-PE (all BD);
- soluble Langerin-FITC (kind gift from M.E. Taylor);
- rhLangerin;
- rhCD44Fc (both R&D);
- Hyaluonic Acid Binding Protein-biotin (Imnosource);
- Isotype-specific goat-anti-mouse Alexa-488, -546 and Streptavidin-488 (all: Invitrogen);
- Hyaluronidase type IV S (Sigma Aldrich);
- Poly(I:C) (Sigma Aldrich);
- TNF-α (R&D);
- MACS CD1a and DC-SIGN microbeads (Miltenyi);
- carboxyfluorescein succinimidyl ester (CFSE, Sigma Aldrich);
- Hydroethidium (HE; Invitrogen);
- dispase II; DNAse I (both: Roche)
- TSA buffer (TSM buffer (T ris buff er (20 mM T ris–HCl, pH 7, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2); TSM); TSA buffer (TSM supplemented with 1% BSA); PBA buffer (PBS supplemented with 0.5-1% BSA and 0.02% Azide); Hyaluronidase treatment buffer (115 mM NaCl; 0.2 mM Na₂HPO₄·2H₂O; 7.7 mM KH₂PO₄; pH5.2)

**Langerhans cell and keratinocyte isolation**
Human skin tissue was obtained from healthy donors undergoing corrective breast or abdominal surgery after informed consent in accordance with our institutional guidelines. Split-skin grafts of 0.3 mm were harvested using a dermatome (Zimmer) and were treated with dispase (1 U/ml) at 37°C for 45 minutes to separate dermis from epidermis. The epidermis was either floated onto medium for 48 hours before mature LCs were harvested from the supernatant. To isolate immature LCs and KCs, epidermis was enzymatically degraded by trypsin and DNAse I, and the single cell suspension was layered on a lymphoprep (Histopaque; Axis-shield) gradient. The pellet contained KCs and the lymphoprep fraction was cultured in the presence of IL-4 and GM-CSF (500 and 800 IU/ml; Biosource/Invitrogen) for 6 days to allow monocyte derived DC (moDC) differentiation. Then, DCs were stimulated for 24 hours with LPS (10 ng/ml) or Poly(I:C) (10 µg/ml).

**FACS analysis**
All cells were washed in PBA or TSA and were incubated with specific antibodies, HABP-bio (5 µg/ml) or isotype controls for 30 minutes at 4°C. Or cells were incubated with soluble langerin (rh langerin; R&D) for 30 minutes at RT. Subsequently cells were washed and incubated with Alexa 488 secondary antibody (5 µg/ml) for 30 minutes at 4°C. Or cells were stained with directly labelled antibodies for 30 minutes at 4°C. Or LCs were fixed with 4% PFA and permeabilized in PBS with 0.5% saponin / 1% BSA, before cells were incubated with primary antibody for 60 minutes at room temperature. Then cells were incubated with isotype-matched secondary antibodies for 30 minutes at room temperature. Finally, the slides were counterstained with Hoechst for 2 minutes. Between all incubation steps, sections were extensively washed with PBS supplemented with Ca²⁺ (pH 7.4). Matched isotype antibodies served as negative control and all controls were essentially blank. Cryosections were analyzed by a Confocal Laser Scanning Microscope (Leica).

**Fluorescent bead adhesion assay**
We performed the fluorescent bead adhesion assay as described before. In short: for HA expression profiling, we coated streptavidine beads with 5 µg of biotinylated goat-a-human-Fc and rhCD44Fc. Cells and beads were co-incubated in TSA for 45 minutes.

**Confocal Laser Scanning Microscopy**
5-µm Cryosections of human skin were air-dried and fixed in acetone for 10 minutes. Sections were preincubated with 10% normal goat serum for 10 minutes before sections were incubated with soluble Langerin-FITC, anti-CD1a, anti-cytokeratin, anti-CD11b or HABP-bio for 1 hour at room temperature. Then sections were incubated with isotype-matched secondary antibodies or Streptavidin-Alexa 488 for 30 minutes at room temperature. Finally, tissue sections were counterstained with Hoechst for 2 minutes. Between all incubation steps, sections were extensively washed with PBS supplemented with Ca²⁺ (pH 7.4). Matched isotype antibodies served as negative control and all controls were essentially blank.

**Immunoprecipitation and immunoblotting**
For immuno-precipitation cells were lysed with RIPA buffer (Cell Signaling) and lysates were preincubated with 10E2...
(anti-langerin). Langerin was precipitated with prot A/G plus agarose beads. Lysates were resolved by SDS-PAGE, and detected by immuno-blotting with goat-anti-langerin or rabbit anti-hyal-2 antibodies. Or cells were lysed in lysis buffer (Cell Signaling) and lysates were resolved by SDS-PAGE, and detected by immuno-blotting with rabbit-anti-hyal-1 and -2 antibodies.

**RNA isolation and quantitative real-time PCR**

mRNA was isolated with an mRNA Capture kit (Roche) and cDNA was synthesized with a reverse-transcriptase kit (Promega). Samples were amplified by PCR with SYBR Green as described before 35. Specific primers were designed with Primer Express 2.0 (Applied Biosystems) for Hyal-2 and housekeeping gene GAPDH. Hyal-2: FW: TGGCCTCCAGACCGCATA; REV: TCCAGATGAACCTGGTGTC; GAPDH: FW: CCAAGTTTCGTGCGTG; REV: GTGTCAAGAGTTGTGGTG . The cycling threshold (CT) value is defined as the number of PCR cycles in which the fluorescence signal exceeds the detection threshold value. Relative expression levels of hyal-2 were calculated from the CT values obtained for both hyal-2 and GAPDH mRNA with the equation \[\text{Relative hyal-2 mRNA} = 2^{(\text{CT}(\text{GAPDH}) - \text{CT}(\text{hyal-2}))}.\]

**REFERENCES**


32. van den Berg, L.M. *et al.* Chapter 5: Langerhans cell-dendritic cell interactions through langerin and hyaluronic acid mediate HIV-1 antigen transfer. In: *The role of C-type lectin receptors in skin immunity.* Amsterdam (2013). submitted for publication