The role of C-type lectin receptors in human skin immunity: immunological interactions between dendritic cells, Langerhans cells and keratinocytes
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CHAPTER 8

GENERAL DISCUSSION
Skin wound repair is an essential physiological process to maintain tissue integrity and homeostasis and therefore wounds need to heal as fast and as functional as possible. Burn injury is the cause of dermal damage and excessive local inflammation, which is associated with poor wound healing and the formation of hypertrophic scars. Therefore we investigated the role of keratinocytes (KCs), Langerhans cells (LCs) and dendritic cells (DCs) in wound healing and inducing immune responses after burn injury. In chapter 3 we showed that both LCs and DCs were functionally disabled after burn injury. In chapter 4 we described that dectin-1 triggering on KCs induced enhanced proliferation and migration, which enhanced wound re-epithelialization.

1.1 Immune response during burn wound healing

Severe burn trauma is characterized by local excessive inflammation of the wound and suppression of the systemic adaptive immune response. Local inflammation delays wound closure and facilitates bacterial infections associated with sepsis and multiple organ failure. LCs and DCs are migratory antigen presenting cells (APCs) inducing adaptive T cell responses. We investigated their role in inducing adaptive immune responses after burn injury. We observed strong decreased T cell stimulatory capacity of both LCs and DCs (chapter 3). In addition we observed that supernatant of burned skin contained a soluble burn factor that enhanced DC-mediated T cell suppression and even suppressed DCs from healthy skin. Thus, LCs and DCs could partially be responsible for the observed suppressed immunity in patients with major burn injuries. Suppression of T cells has already been described in patients. Peripheral blood lymphocytes isolated from burn patients remained anergic to T cell receptor triggering. Our data provide evidence for a role of LCs and DCs in establishing T cell anergy and subsequent suppressed T cell responses observed in patients with burn trauma. However, the mechanism underlying skin DC-mediated T cell suppression remains to be investigated. Phenotypically, there were no differences in expression levels of co-stimulatory molecules between DCs from burned skin and unburned skin. In addition, the number of cells migrating from skin and unburned skin was similar, suggesting DCs were equally functional (chapter 3). Co-inhibitory molecules expressed by DCs of the B7 family, such as B7-H1, B7-H4 and B7-DC, or the production of indoleamine-2,3-dioxygenase (IDO), which induces tryptophan starvation of T cells, might play a role in T cell suppression. However, we did not observe up-regulation of B7-H1 or B7-DC on the cell surface of skin DCs from burned skin (chapter 3) and the production of IDO remains to be investigated. Our data suggest that restoring the function of LCs and DCs could prevent T cell suppression and therefore might be beneficial for patients with major burn trauma in restoring systemic tissue homeostasis and to accelerate wound closure (chapter 4).

In addition to cellular dysfunction of LCs and DCs from burned skin, the supernatant of burned skin contained a heat-stable soluble factor that enhanced DC-mediated T cell suppression (chapter 3). ‘Burn toxins’ have been described, which are lipid-protein complexes isolated from burned murine skin. This burn toxin induced
liver damage and had lethal effect after intraperitoneal injection into recipient mice. Although the exact composition of the burn toxin has not been identified, early excision of the burned area in murine models leads to restoration of the T cell responses to viruses and bacteria. Therefore, the burn toxin most likely is a cytosolic component or damage associated molecular pattern (DAMP) that is forced into the surrounding tissue after burn injury, which exerts a suppressive effect on skin DCs and thus on adaptive immunity.

Prolonged inflammation and inflammatory cytokines have been associated with delayed wound closure. Therefore we targeted C-type lectin receptors (CLRs) in skin to interfere with the immune response to enhance wound closure. CLRs are conserved receptors recognizing carbohydrate structures. The CLR dectin-1 is expressed by LCs, DCs and keratinocytes (KCs) and recognizes the 1,3-linked fungal beta-glucan curdlan. In DCs, dectin-1 triggering results in the production of IL-6 and other anti-fungal cytokines. However, in KCs dectin-1 stimulation induced increased proliferation and migration leading to increased wound closure in an ex vivo wound healing model (chapter 4). In addition, we speculate that dectin-1 activation of LCs and DCs might induce restoration of LC and DC function in burned skin (chapter 3). Carbohydrate structures can easily be applied in topical crèmes covering the wound. Thus, manipulating the immune response via CLRs could be beneficial for wound healing.

PART II - A NOVEL FUNCTION FOR LANGEVIN AS CELLULAR ADHESION RECEPTOR

The mammalian CLR family is divided into 17 subgroups and type II, IV and V CLRs are expressed on immune cells. CLRs expressed by immune cells mainly function as pathogen recognition receptors (PRRs), whereas the majority of CLR-family members function as cellular attachment receptors. Langerin, DC-SIGN (both type II) and dectin-1 (type V) are CLRs present on human LCs and DCs recognizing carbohydrate structures derived from viruses, bacteria and fungi (chapter 2). For DC-SIGN and dectin-1 recognition of autologous ligands has been described. DC-SIGN interacts with ICAM-2 and ICAM-3 in the immunological synapse formed upon interaction with T cells and with Mac-1 (CD11b/CD18) and CEACAM1 on neutrophils. Dectin-1 interacts with an unknown ligand on T cells. For human langerin recognition of self-glyco-proteins has been predicted, but has not been described so far. In this manuscript we identified the cellular ligand for langerin, which is the glycosaminoglycan hyaluronic acid (HA) expressed on DCs (chapter 5) and KCs (chapter 6).

2.1 HA as cellular ligand for langerin

Human langerin has high affinity for mannose-, fucose- and GlcNAc-mono- and polysaccharides. Langerin efficiently bound the GlcNAc-containing HA polymer, which is abundantly present in epidermis and dermis (chapter 5 and chapter 6). We identified langerin as attachment receptor for HA-expressing KCs and DCs (Fig 1A). The interaction between langerin and HA is most probably not static, but
Figure 1: Overview of LCs, DCs and KCs in skin
(A) LCs reside in the epidermis in close proximity to HA expressing KCs. In dermis, HA expressing DCs are present. LCs and KCs interact via langerin-HA. (B) LCs mature upon encountering pathogens and upregulate hyaluronidase-1 and -2. Hyal-2 cleaves HA from surrounding KCs, which allows the LC to migrate towards the dermis. (C) In dermis, LCs and DCs cluster via langerin-HA. This interaction enables antigen transfer from LC to DC and induces DC and LC maturation. (D) Mature DCs upregulate hyal-2, which cleaves HA from the cell surface and downregulates HA expression. LCs and DCs abrogate clustering. (E) DCs cross-present antigens to CD8\(^+\) T cells. DC: dendritic cell; HA: hyaluronic acid; KC: keratinocyte, LC: Langerhans cell.

rather dynamic. Langerin is a recycling receptor that recycles from the cell surface to endocytic recycling compartments of LCs across the cell surface, which can enable dynamic interactions with either HA or pathogenic carbohydrate structures. Furthermore, HA is also expressed by fibroblasts, endothelial cells and activated T cells, which suggests LCs interact with a broad spectrum of cells in the body. Therefore, strict regulation of HA expression is required on cells that interact with langerin-expressing LCs, as described in chapter 6.

HA is a highly conserved glycosaminoglycan in vertebrates that forms tertiary structures controlling water homeostasis, structural integrity and the sequestration of free radicals. Expression and degradation of HA is regulated by hyaluronidases. Vertebrates are equipped with genes encoding 6 hyaluronidases, however catabolism of HA in somatic tissues is regulated for the greater part by hyaluronidase-1 (hyal-1) and hyal-2. Hyal-2 is GPI-anchored to the cell membrane and cleaves HA high molecular weight (HMW; > 1*10\(^5\) kDa) into fragments of low molecular weight (LMW; ~20 kDa). LMW fragments are immuno-stimulatory and induce signalling via CD44, TLR4 and TLR2 in DCs and macrophages. HA LMW fragments are endocytosed and
further degraded intracellular by hyal-1. Knocking out of gene HYAL2 is lethal to mice \(^{29}\), indicating HA regulation is indispensable for systemic homeostasis.

In the epidermis, LCs interact with KCs via langerin and HA (Fig 1A). LCs are retained in epidermis and we investigated whether langerin-HA interaction is sufficient to maintain LCs in epidermis and how this interaction is regulated. We described that LCs upregulated both hyal-1 and hyal-2 upon maturation (chapter 6). Hyal-2 cleaved and degraded HA from the KC surface, which subsequently enabled LC migration (Fig 1B). Hyal-1 expressed by LCs supposedly cleared the HA LMW fragments from the epidermal surrounding, minimizing local inflammation. LCs express TLR2 \(^{32}\) and therefore HA LMW fragments might induce maturation of LCs. The HYAL1 gene has a transcription factor binding site for NF-κB, Egr-1 and AP-2 \(^{33}\). NF-κB is activated upon maturation by TLR or CLR stimulation \(^{15,34,35}\) and therefore initial hyal-1 expression in mature LCs could be NF-κB dependent. For the HYAL2 gene transcription regulation is not known. Thus, our data provide strong evidence that LC binding to KCs and LC migration from epidermis is regulated by maturation of LCs and subsequent expression of hyal-1 and hyal-2.

### 2.2 LC-DC clustering via langerin-HA

Although langerin expression is downregulated upon LC maturation, the expression was sufficient to induce strong clustering with DCs in dermis (Fig 1C). This is further supporting that HA downregulation on KCs abrogated LC-KC interaction and not a decrease in langerin expression upon maturation. We showed that LCs induced maturation of DCs and thereby induced the expression of hyal-2 on DCs (Fig 1D). Hyal-2 cleaved HA from the DC cell surface, which abrogated langerin binding and LC-DC clustering (chapter 5 and chapter 6). In contrast to LCs, DCs did not upregulate hyal-1 upon maturation, suggesting either constitutive expression is sufficient to clear HA LMW from the surrounding, or increased presence of short HA fragments can locally enhance inflammatory responses by other resident immune cells, such as macrophages. HA is extruded through the cell membrane into the extracellular matrix where HA HMW forms tertiary structures that are important for the structural integrity of the tissue \(^{36}\). Therefore, the expression of HA on the cell surface of DC might facilitate DC anchoring in the dermis. TLR3 and TLR4 ligands Poly(I:C) and LPS induced upregulation of hyal-2 in DCs, suggesting downregulation of HA is not only needed to abrogate LC-DC clustering, but could also be necessary for DC migration.

In contrast to DCs, immature LCs are not efficient in cross-presenting exogenous pathogens \(^{37-40}\). Antigen transfer between DC subsets has been suggested in murine models, where it was shown that CD8\(^+\) T cell priming is not depended on herpes simplex virus (HSV)-antigen presentation by LCs \(^{41}\), but depends on lymph node resident DCs \(^{41}\). In murine models, skin DCs migrate faster to the lymph nodes compared to LCs \(^{42,43}\), and therefore antigen transfer would enhance antigen spreading and the efficiency of inducing adaptive immune responses. We showed that interaction between human LCs and DCs enabled HIV-1 antigen transfer for cross-presentation to CD8\(^+\) T cells (Fig 1E and chapter 5). Thus, the transfer of antigens between LCs and
DCs most probably is an efficient system to induce rapid adaptive immune responses to epidermal derived pathogens. However, antigen transfer to DCs is not enough for inducing an immune response, since correct activation of DCs by PAMPs is required for upregulation of co-stimulatory molecules. We showed that LC-DC interaction not only led to transfer of antigens but also induced maturation of DCs (chapter 6). Preliminary data suggest that LCs boost the DC-mediated CD4+ T cell response (data not shown), suggesting LCs enhance the immunostimulatory capacity of DCs. Therefore, antigen transfer between LCs and DCs might be a highly efficient system to boost immune responses to harmful skin-derived pathogens.

The mechanisms underlying LC-DC antigen transfer are not unravelled yet. We showed that interaction between LCs and DCs via langerin and HA is indispensable for antigen transfer between these two subsets. Since HIV-1 is not cytolytic to LCs (data not shown) transfer of apoptotic LC bodies to DCs is unlikely. Trogocytosis typically occurs between APCs and T cells, by transferring peptide-MHC complexes from APC to T cell 44, 45. However, trogocytosis of peptide-MHC class I complexes between LCs and DCs can be ruled out, since LCs did not cross-present HIV-1 on MHC class I to T cells and, in addition, we mismatched LC/T cell HLA*A02 typing. Exosomes represent a specific subtype of secreted membrane vesicles, carrying adhesion molecules, signalling molecules and cargo of the donor cell 46. By electron microscopy we observed LCs are highly positive for HIV-1 positive exosomes (data not shown). Therefore we suggest LCs partly degrade HIV-1 and transfer exosomes or particles to DCs in the immunological synapse formed between LCs and DCs. Still it remains speculative about the mechanisms of antigen transfer between LCs and DCs, and further work is required to clarify this issue.

**PART III - LC IMMUNITY AGAINST HIV-1**

LCs not only reside in epidermis of skin, but also localize in vaginal epithelium and foreskin and therefore are the first immune cells encountering HIV-1 upon sexual intercourse 47, 48. LCs take up HIV-1 via langerin into Birbeck granules (BGs) 47, which are unique langerin+ organelles 49. Langerin-mediated uptake of HIV-1 leads to degradation of the virus and prevents transmission to T cells; therefore, LCs have protective function against HIV-1 47. Multiple hypotheses exist for the origin and function of BGs, and the current vision about BGs is that it is part of recycling endosomal compartments 24, 50. Langerin is internalized into BGs where it accumulates into Rab11+ early endosomal compartments before it recycles back to the membrane 24, 51. Therefore, it has been assumed that langerin is part of the clathrin-mediated internalization route 24 and we challenged this by identifying BGs as caveolar vesicles, being part of the caveolin-1-mediated endocytosis pathway (chapter 7). In the next paragraphs, we will discuss the implications of these findings for the understanding of BGs in the degradation and antigen-presentation route of HIV-1 in LCs (chapter 5).
3.1 Langerin/Caveolin-1 internalization pathway

Caveolar endocytosis is caveolin-1 dependent and occurs in lipid rafts. Besides cellular uptake via clathrin-coated pits, endocytosis via lipid rafts is a major endocytosis route. Caveolae are caveolin-1 positive invaginations of the membrane that can bud off and form caveolar vesicles. Caveolar uptake in lipid rafts is slower and clearly distinct from clathrin-coated pits; however, the intracellular trafficking partially overlaps. Both langerin and caveolin-1 have recently independently of each other been associated with Rab11a and Rab11-FIP2 recycling endosomal trafficking. Our data showed that BGs are caveolin-1 positive and therefore link these recent findings by identifying BGs as caveolar vesicles (Fig 2 and chapter 7). Caveolin-1 is targeted via late endosomes to lysosomes and these routing has also been observed for langerin mediated uptake. Caveolar vesicles have clover-like structures in langerin-negative cells. Apparently the unique combination of langerin and caveolin-1 in LCs induces the formation of tennis-racquet-like shaped BGs (Fig 2). Thus, in chapter 7 we linked caveolae to the uptake receptor langerin and BGs.

Langerin mediates uptake of HIV-1 in LCs and targets the virus to BGs. We showed that blocking caveolar uptake prevents HIV-1 internalization in LCs (chapter 7). Moreover, blocking this internalization route increased HIV-1 integration in the LC-genome (Fig 2). Thus, caveolar uptake and subsequent routing to lysosomal structures protects LCs against HIV-1 infection. These data suggest that there is a second langerin-independent route ‘Route X’ of HIV-1 uptake or HIV-1 fusion that facilitates HIV-1 integration in LCs (Fig 2). This second route could also be implicated in increased HIV-1 infection of mature LCs, which has been ascribed to langerin downregulation. Genital co-infections with sexual transmitted diseases such as HSV-2, increased HIV-1 infection of LCs.

Figure 2: HIV-1 is taken up in caveolin-1 positive Birbeck granules

Langerin and caveolin-1 mediate the uptake of HIV-1 into Birbeck granules. HIV-1 is targeted towards lysosomal compartments that are CD63 and LAMP-2 positive. This degradation pathway overlaps with the pathways described for langerin and caveolin-1. Integration into the host genome increases when the caveolar pathway is blocked, which suggests a second endocytosis route (‘Route X’) for HIV-1 that is not protective against HIV-1 infection.
Decreased langerin-mediated uptake of HIV-1 due to saturation of langerin and downregulation of langerin has been used as explanation for the increased infection of LCs. Our data suggest that langerin-independent uptake of HIV-1 by an unknown pathway could account for this increased infection. Therefore we suggest that langerin-mediated HIV-1 uptake into caveolar vesicles is the most efficient pathway for HIV-1 internalization and degradation in LCs. As long as this route is intact, LCs have protective function against HIV-1.

3.2 LC mediated anti-HIV-1 adaptive immunity

Virally infected cells will present endogenous antigens onto MHC class I to cytotoxic T cells. However, APCs have the capacity to cross-present exogenous viral proteins onto MHC class I for cytotoxic T cell activation, without the need for direct infection of the cells. Since LCs are not efficiently infected with HIV-1 and have anti-HIV-1 functions, we expected LCs to induce HIV-1 specific cytotoxic T cells via cross-presentation. However, LCs were not able to cross-present HIV-1 to CD8+ T cells, whereas DCs were very efficient in cross-presenting HIV-1 to CD8+ T cells (chapter 5). Our data showed that LC-DC clustering facilitated antigen transfer; LCs transferred HIV-1 antigens to DCs for cross-presentation. This division of labour has also been hinted at in murine models. In contrast to LCs, murine dermal CD103+ DCs are efficiently cross-presenting skin derived antigens. Depletion of LCs from murine epidermis exacerbates HSV pathogenicity indicating LCs locally have an important anti-viral role. However, CD8+ T cell priming is not depended on HSV-antigen presentation by LCs, but depends on lymph node resident DCs. In chapter 5 we described division of labour among human LCs and DCs. Locally immature LCs are protective against HIV-1 infection, however our data strongly suggest LCs rely on dermal or submucosal DCs for cross-presentation and subsequent induction of adaptive anti-viral immune responses.

This raises the question whether LCs are intrinsically incapable of cross-presenting antigens, or whether the uptake and routing of the pathogen is determining cross-presentation. It could be that HIV-1 uptake and degradation via langerin/caveolin-1 is highly efficient and therefore excludes cross-presentation. Immature (CD34+ monocyte-derived) LCs are incapable of cross-presenting exogenous tumour proteins or virus like particles, whereas mature (CD34+ monocyte-derived) LCs stimulated with IFN-γ or CD40-ligand cross-present exogenous antigen. Maturation of LCs downregulates langerin expression and might target pathogens to the ‘Route X’ endocytosis pathway suggested in paragraph 3.1 (Fig 2), which might allow for cross-presentation. Therefore we postulate the main specialization of immature skin LCs is to clear pathogens from the surrounding preventing infection. This might restrict their ability for cross-presentation, since any leakage to the cytoplasm might allow infection.

CONCLUDING REMARKS

In this manuscript we have investigated the role of LCs, DCs and KCs in maintaining and repairing the human skin barrier and preventing infections. KCs provide the mechanical barrier against pathogens, whereas LCs and DCs provide the immunological
barrier against pathogens. We have investigated the role of KCs in wound healing after burn injury, and the role of LCs and DCs in immune responses mounted upon burn injury. Cellular interactions between LCs and KCs are important for maintaining tissue integrity and for retaining LCs in the epidermis, while clustering between LCs and DCs enable efficient induction of adaptive immune responses against harmful epidermal or mucosal pathogens, such as HIV-1. Furthermore we have identified the origin of BGs, which are implicated in protection against HIV-1. Understanding these mechanisms and the complex interactions between different cell subsets can contribute to new therapeutic strategies against microbial and viral infections and enhance the quality of wound healing.

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