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 3

Burn injury suppresses human dermal dendritic cell and Langerhans cell function

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Abstract

Human skin contains epidermal Langerhans cells (LCs) and dermal dendritic cells (DCs) that are key players in induction of adaptive immunity upon infection. After major burn injury, suppressed adaptive immunity has been observed in patients. Here we demonstrate that burn injury affects adaptive immunity by altering both epidermal LC and dermal DC function. We developed a human *ex vivo* burn injury model to study the function of DCs in thermally injured skin. No differences were observed in the capacity of both LCs and dermal DCs to migrate out of burned skin compared to unburned skin. Similarly, expression levels of costimulatory molecules were unaltered. Notably, we observed a strong reduction of T cell activation induced by antigen presenting cell (APC) subsets that migrated from burned skin through soluble burn factors. Further analyses demonstrated that both epidermal LCs and dermal DCs have a decreased T cell stimulatory capacity after burn injury. Restoring the T cell stimulatory capacity of DC subsets might improve tissue regeneration in patients with burn wounds.

Introduction

Skin is part of the integumentary system which is the largest organ system of the mammalian body. Skin wound repair is therefore an essential physiological process to maintain tissue integrity and homeostasis. Wound healing is a highly organized process involving different phases: inflammation, tissue formation and tissue remodeling. Immediately after skin wounding, a fibrin clot forms as temporary barrier and leukocytes start infiltrating the wound. Cells present at the ruptured site activate resident immune cells, such as mast cells, antigen presenting cells (APCs) and γδ-T cells, that release chemokines and cytokines to attract other immune cells. Neutrophils and macrophages are recruited which results in the inflammatory response to kill invading microorganisms. The level of inflammation directs the quality of wound repair: the immune response needs to be down regulated to achieve successful tissue regeneration. Finally, skin is re-epithelialized by keratinocytes while fibroblasts and myofibroblasts help to close the wound. Virtually every dermal injury heals with a scar as endpoint.

Exaggerated inflammation during wound healing is associated with non-healing chronic wounds, the formation of hypertrophic scars and keloids. Burn injuries lead to dermal damage and excessive inflammation that impairs the ability of the skin to regenerate. Therefore hypertrophic scarring is a phenomenon frequently observed after thermal dermal injury. The excess inflammation observed after burn injury increases the concentration of potential profibrotic cytokines like Transforming Growth Factor-β (TGF-β), platelet-derived growth factor (PDGF) and Interleukin-4 (IL-4). Under influence of TGF-β1 and β2 fibroblasts differentiate into myofibroblasts that lead to excessive extracellular matrix deposition and fibrotic tissue after burn injury. Systemically, patients with burn wounds suffer from suppressed adaptive immunity that can lead to multiple organ failure or sepsis. This might partly be due to T-helper 2
cell skewing (Th2) as a result of the high levels of TGF-β and IL-4\(^{10,11}\) that leads to suppressed T-helper 1 (Th1) function\(^{12}\). At the site of burn, necrotic tissue (eschar) could also exert effects on the immune response during wound healing. Eschar might provide a nutritious substrate for (opportunistic) micro-organisms. It has been shown in mice that direct removal of the eschar after burn results in restoration of the immune response\(^{13,14}\).

Dendritic cells (DCs) are professional APCs that are present in skin and monitor their surrounding for pathogens; upon encountering antigen DCs migrate towards the lymph node and present the antigen to T cells. Since DCs bridge the innate and adaptive immune system\(^{15}\) we hypothesized an important role for skin DCs in initiating the immune response after burn. In resting human skin, mainly three populations of APCs have been described: the epidermal Langerhans cells (LCs), the dermal DCs and macrophages\(^{16}\). LCs can be distinguished by high CD1a expression\(^{17}\) and the presence of the C-type lectin Langerin\(^{18}\). In the dermis, CD1a\(^{+}\)CD11c\(^{-}\) and CD1a\(^{-}\)CD11c\(^{+}\) DCs are present\(^{19}\). CD1c (BDCA-1) marks the CD1a\(^{+}\)CD11c\(^{+}\) dermal DCs while the scavenger receptor CD163 is present on the CD1a\(^{-}\)CD11c\(^{-}\) macrophages\(^{17}\). It has been described that DCs are the most potent immune inducers compared to macrophages\(^{15}\).

Little is known about the role of human LCs and DCs in the inflammatory response observed after burn injury. Therefore we developed a human ex vivo burn injury model to study the function of LCs and DCs in thermally injured skin. Notably, we observed a strong suppression of the T cell stimulatory function of both LCs and DCs after burn injury; whereas migration and expression levels of costimulatory molecules were unaltered. Further analysis showed that the soluble fraction induced by burn injury suppresses not only DCs from injured skin, but also from healthy tissue. Thus we demonstrate that dermal CD1a\(^{+}\) and CD1a\(^{-}\) DCs and LCs migrated from burned skin are the main migrating APC populations with a reduced capacity to induce T cell proliferation. Better understanding the role of DCs in the inflammatory phase during wound healing after burn injury might give better inside in the hypertrophic scarring process and the observed suppressed systemic immune system in patients.

**RESULTS**

**Histology of healthy and burned skin**

We investigated the presence of antigen presenting cells in healthy human tissue. In concordance with literature we were able to distinguish three subsets of APC: the epidermal Langerin\(^{+}\) Langerhans cells (Fig 1a) the dermal CD1a\(^{+}\) dendritic cells and the dermal CD1a\(^{-}\) CD163\(^{+}\) macrophages (Fig 1b). Next we investigated the effect of burn injury on DC function. We developed an adjustable heating system, the Human Ex vivo Adjustable Temperature regulating - Machine (HEAT-M), that was used to induce controlled burns of a specific size at a specific temperature (Fig 1c). A split-skin graft of 0.3 mm was cut into pieces of 1 cm\(^2\) and was burned at a surface of 2 mm by 10 mm at 95°C for 10 seconds. Healthy human skin consisted of a solid keratinized epidermis with the looser, collagen containing, dermis underneath it (Fig 1d). Directly after
introducing the burn wound onto the skin, the skin blistered and the epidermis detached locally (Fig 1e). Burned skin was cultured for 24 hours and we observed further detachment of the epidermis up to 40% of the surface area (Fig 1f) while we did not observe this in unburned tissue (data not shown). LCs, DCs and macrophages were depleted from the burn site, but were still present in surrounding tissue (data not shown). These data strongly suggest that the injury is a full thickness burn wound as has been described before.22

Migration and maturation of DC subsets not affected by burn injury

In vivo DCs migrate towards the lymph node upon activation after encountering antigen. To mimic cell migration, ex vivo skin grafts were floated onto medium and migrating antigen presenting cells were analyzed (Fig 2a). HLA-DR was used as marker for APCs migrating from both burned and unburned skin. Three major APC populations migrated from skin after 24 hours: CD1a⁺/Langerin⁺ cells, CD1a⁺ cells and CD1a⁻ cells (Fig 2b left panels, middle panels). All HLA-DR⁺ cells were also positive for
CD11c (data not shown). No differences in population size were observed between cells from burned skin compared to unburned skin suggesting that burn injury did not affect migration. 3 - 4.5% of the CD1a+ population consisted of Langerhans cells since they expressed Langerin (Fig 2b middle panel). The majority of migrating cells were dermal CD1a+ DCs (Fig 2b) that express CD11c (data not shown) but no Langerin. No CD163 expression could be detected on migrated cells, indicating that dermal macrophages did not migrate from skin (Fig 2b right panels).

Fig 2: Three populations of dendritic cells migrate out of skin
Floating of split skin grafts onto medium induces skin DC migration (a). Three populations migrate out of unburned as well as burned skin after 24 hours. Based on HLA-DR expression CD1a negative, CD1a positive and CD1a positive/Langerin positive cells can be identified (b, left panels, middle panels). No CD163 expression could be detected on the migrating cells (b, right panels) suggesting macrophages reside in the skin. This experiment is representative for three donors; one representative experiment out of three is shown.
Activation of DCs and subsequent migration results in upregulation of MHCII and costimulatory molecules such as CD80, CD86 and CD40. To investigate whether cells from burned skin displayed differences in phenotype and migrational behavior, the number of live migrating cells as well as different surface markers was determined after 24 hours. Dead cells were excluded by 7AAD and Annexin-V staining (data not shown). Burn injury did not affect the number of viable cells migrating out of 1 cm² of skin (Fig 3a). Remarkably, no differences were observed in the expression-level of costimulatory markers CD80, CD83, CD86, HLA-DR and CD40 (Fig 3b). These data suggest that APCs migrating from burned skin have the prerequisites to induce an effective immune response. Thus, migration as well as activation phenotype of LCs and DCs was not affected by burn injury.

**Fig 3: Both migratory capacity and activation phenotype of DC subsets is not changed after burn injury.** The number of living cells migrating from 1 cm² burned skin does not differ from the number of unburned skin (a). The expression levels of costimulatory molecules CD80, CD83, CD86 and CD40 as well as the MHCII molecule HLA-DR are similar on APCs migrated from burned skin compared to unburned skin (b). This experiment is representative for more than five donors; one representative experiment out of five is shown. Error bars represent standard errors of tetraplicates.

**DCs from burned skin have decreased T cell activating capacity**

T cell activation by mature DCs is essential in initiating effective immune responses against invading pathogens. Therefore we compared the HLA-DR⁺ DCs from burned and unburned skin in a mixed leukocyte reaction (MLR) with allogeneic PBLs. The total pool of migrated HLA-DR⁺ cells was quantified, normalized and added to T cells in different ratio’s (Fig 4a). DCs from unburned skin efficiently induced T cell proliferation (Fig 4a). Notably, DCs migrated from burned skin induced less T cell proliferation compared to unburned skin (Fig 4a). The suppression was due to dysfunction of DCs since extensively washing of DCs before addition of T cells did not restore T cell activation (Fig 4a). However, the difference in T cell proliferation between the washed and unwashed condition indicated that burned skin produced soluble factors that enhanced suppression locally. In order to investigate whether indeed soluble factors affect DC function, we cultured mature monocyte-derived DCs (moDCs) with the
soluble fractions from unburned and burned skin. Notably, treatment of mature moDCs with the soluble fraction from burned but not unburned skin significantly reduced T cell activation (Fig 4b), demonstrating that soluble factors affect DC function. Similarly, the T cell stimulatory capacity of DCs isolated from skin was also decreased when treated with the soluble fraction of burned skin (Fig 4b). The soluble fraction of burned skin did not suppress proliferating T cells (activated with IL-2/PHA; data not shown) indicating that it is a direct effect on DC function.

IL-10 and TGF-β1 are cytokines associated with T cell suppression. The soluble fractions were heat inactivated (HI) for 15 minutes at either 75°C or 95°C to denature proteins. The suppressive effect could still be observed indicating that the suppressive agent is not a heat sensitive protein (Fig 4b). In addition, IL-10 levels were not detectable by ELISA in the soluble fractions (< 8 pg/ml; data not shown). TGF-β1 is a heat-stable cytokine and to exclude TGF-β1 as suppressive agent it was blocked by a neutralizing anti-TGF-β1 antibody. However, no restoration of the T cell response could be detected for both moDCs and skin-derived DCs (Fig 4c), whereas the antibody did restore TGF-β1-mediated T cell suppression (data not shown). These data strongly suggest that DCs from burned skin have a reduced capacity to induce T cell activation and soluble factors present in burned skin affect DC function.

Fig 4: Dysfunctional DCs from burned skin induce less T cell proliferation

Dendritic cells from burned skin induced less T cell proliferation in a mixed leukocyte reaction with allogeneic T cells compared to unburned skin (a). After extensive washing, burned DCs still showed less induction of T cell proliferation indicating the suppression is cell mediated. There is an additional effect of the culture medium from burned skin since T cell proliferation is attenuated even further if cells are not washed (a). LPS-matured moDCs and skin DCs from unburned skin show lower T cell activation in the presence of the burned supernatant (b). The burned fraction suppresses moDCs in their ability to induce
**Fig 4 continued** T cell proliferation and this effect cannot be reverted by heat inactivating (HI) the fraction (b) (a, b, one experiment out of four is shown). TGF-β₁ was blocked with anti-TGF-β₁ antibody but it did not restore the T cell response (representative for one donor) (c). Error bars represent standard deviation of triplicates. CPM: counts per minute.

**Fig 5: Burned dermal DCs and LCs decrease T cell proliferation.**
Cells were selected for their CD1a expression (a) by CD1a-magnetic bead selection. Cells were analyzed for CD80, CD83, CD86, CD40 and HLA-DR expression that remained unchanged among different conditions (b). Both CD1a⁺ and CD1a⁻ dermal DCs from burned skin altered T cell proliferation in an allogeneic MLR (c). LCs migrated from burned epidermal sheets were also decreased in their capability to induce T cell proliferation (d). Error bars represent standard deviation of triplicates. These experiments are representative for three donors; one out of three is shown. CPM: counts per minute.

**Both DC subsets from burned skin are dysfunctional**
Next, migrated cells were sorted by their CD1a expression using magnetic beads (Fig 5a) into CD1a⁺ and CD1a⁻ DCs. The expression levels of CD80, CD83, CD86, HLA-DR and CD40 were similar between the CD1a⁺ and CD1a⁻ dermal DC subsets and did not differ from those obtained from unburned or burned skin (Fig 5b). Next, the antigen presentation capacity of the DC populations from burned and unburned skin was compared in an MLR with allogeneic T cells. The CD1a⁺ unburned dermal DCs were more efficient in activating T cells compared to the CD1a⁻ dermal DCs. Notably, both CD1a⁺ and CD1a⁻ DCs from burned skin led to decreased T cell proliferation (Fig 5c). To exclude that suppression is due to dead cells or residues in the negative fraction,
the experiment was repeated by sorting cells into CD1a positive and negative fractions. The results obtained were comparable to those with beads isolation (data not shown). To investigate the capacity of LCs to induce T cell proliferation. 0.3 mm split skin grafts were burned and epidermis and dermis were separated from each other. Epidermal sheets were floated onto medium for 24 hours and LCs migrated out. Viable cells were quantified, normalized and added to T cells in different ratios (Fig 5d). LCs from burned skin induced less T cell proliferation compared to LCs from unburned skin (Fig 5d). Thus, burn injury affects al DC subsets in skin by decreasing their T cell activation capacity, which might contribute to burn related immunosuppression observed in patients.

CONCLUSIONS

Our results suggest that burn injuries influence the T cell response elicited by human skin DCs and LCs. We observed decreased T cell activation by both LCs and DCs after burn injury. Next to this cellular suppression, we observed that the soluble fraction from burned skin suppressed moDCs and skin DCs to induce T cell proliferation. Thus these data indicate that DCs from burn wound areas have lower T cell proliferation inducing capacities compared to unburned areas. In addition local burn factors might enhance suppression by influencing local resident APCs and immune cells infiltrating the injured area via the blood.

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AUTHORSHIP

LMvdB designed, executed and interpreted most experiments and wrote the manuscript. MAWPdJ and LdW helped designing most experiments. MMWU helped with setting up the ex vivo burn injury model. TBHG supervised all aspects of this study. The authors state not conflict of interest.
**Material and Methods**

**Antibodies and Reagents**

The following antibodies and reagents were used: anti-CD40 (BD Bioscience, San Jose, CA), anti-CD163 (EdHU-1, kind gift from Prof. Dr. C.D. Dijkstra, VU University Medical Center, Amsterdam, the Netherlands), anti-CD1a (Santa Cruz, Heidelberg, Germany), anti-TGF-β (5 μg/ml; R&D systems, Abingdon, United Kingdom), anti-CD80-PE, anti-CD86-PE, anti-HLA-DR-PE (all BD Bioscience, San Jose, CA), anti-CD83-PE (Beckman Coulter, Woerden, the Netherlands), anti-CD1a-FITC (BD Bioscience, San Jose, CA), anti-CD1a-PE (Abcam, Cambridge, United Kingdom), DCGM4-PE (anti-Langerin; Immunotech, Praha, Czech Republic), 10E2 (anti-Langerin; Dako, Glostrup, Denmark), anti-CD163-PE (eBioscience, San Diego, CA), Goat-anti-Mouse Alexa 488 (Invitrogen, Breda, the Netherlands), Isotype control anti-mouse IgG1, IgG2a (all Sanbio, Uden, the Netherlands) normal mouse serum, [3H]-thymidine (Amersham Biosciences, Uppsala, Sweden), dispase (Invitrogen, Breda, the Netherlands). The following buffers were used: TSM buffer (Tris buffer (20 mM Tris-HCL, pH 7, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2); TSM), TSA buffer (TSM supplemented with 0.5% BSA), PBA buffer (PBS supplemented with 0.5% BSA and 0.02% Azide).

**Skin burning**

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, Utrecht, the Netherlands) and were cut into pieces of 1 cm². Skin was burned by using the Human Ex vivo Adjustable Temperature regulating-Machine (HEAT-M). The HEAT-M consists of a copper device (2x10 mm) attached to the tip of an adjustable soldering iron (HQ/Nedis, ’s Hertogenbosch, the Netherlands; voltage converter, HQ/Nedis, ’s Hertogenbosch, the Netherlands). The HEAT-M was heated up to 95°C and applied for 10 seconds at the epidermal side of the skin, without exerting pressure. Skin samples were dermis-down floated onto Iscoves Modified Dulbecco’s Medium (IMDM), 10% FCS, pen/strep (10 U/ml and 10 μg/ml, respectively; Invitrogen, Breda, the Netherlands) and gentamycin (20 μg/ml; Centrafarm, Etten-Leur, the Netherlands) for 24 hours. Or the skin samples were treated with dispase (2 mg/ml) at 37°C for 45 minutes to separate dermis from epidermis and the epidermis was floated onto medium. Skin grafts were embedded in Tissue-Tek (Ted Pella, Redding, CA) and snap-frozen in liquid nitrogen directly after burning or after 24 hours of culturing and subsequently used for immunohistochemical analysis. After 24 hours, migrated cells were harvested from the medium and were layered on a lymphoprep (Axis-shield, Heidelberg, Germany) gradient. Subsequently, cells were analyzed by FACS analysis or used in a T cell proliferation assay. Conditioned culture medium of unburned and burned skin was collected and added to monocyte derived DCs (moDCs) and skin DCs as soluble fraction.

**Immunohistochemical staining**

5-μm Cryosections were air-dried and fixed in acetone for 10 minutes. Sections were stained with haematoxylin and eosin. Or sections were blocked with En Vision dual enzyme block (Dako, Glostrup, Denmark) and preincubated with 10% normal goat serum before sections were incubated with primary antibody (IgG2a) for one hour at room temperature. Sections were incubated with EV-goat-anti-rabbit/mouse HRP (Dako, Glostrup, Denmark) for 30 minutes. Peroxidase labeling was visualized by En Vision 3,3-diaminobenzidine (EV-DAB, Dako, Glostrup, Denmark). Next, sections were blocked with normal rabbit serum + anti-mouse IgG2a and subsequently incubated with the second primary antibody (IgG1) for one hour, followed by alkaline phosphatase conjugated goat-anti-mouse IgG1 (AbD Serotec, Dusseldorf, Germany). Sections were washed in 0.2M Tris-HCl buffer, pH 8.5 and alkaline phosphatase was visualized by Liquid Permanent Red (Dako, Glostrup, Denmark). Finally, tissue sections were counterstained with haematoxylin (Klinipath, Duiven, the Netherlands) for 30 seconds. Between all incubation steps, sections were extensively washed with PBS (pH 7.4). Matched isotype antibodies served as negative control and all controls were essentially blank.

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**FACS analysis**

All cells migrated from 1 cm² were washed in PBA and incubated with specific antibodies (5 μg/ml) or isotype controls for 30 minutes at 4°C and followed by an incubation with Alexa 488 secondary antibody for 30 minutes at 4°C. Subsequently, cells were blocked with 10% normal mouse serum for 10 minutes and incubated with directly labelled antibodies. Cells were washed and binding was measured using flow cytometry.
Magnetic bead cell separation
Cells migrated from unburned or burned skin were harvested and layered on a Lymphoprep gradient prior to CD11c separation by MACS magnetic microbeads (MACS, Miltenyi biotec, Utrecht, the Netherlands) following the manufacturer's protocol. Cells were resuspended in conditioned medium.

Statistical analysis
A student's t-test was used to evaluate the differences between two groups. Experiments were performed in triplicates and are representative for at least three independent donors. p<0.05 was considered significant in all analyses.

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