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Rapid Communication

Dectin-1 activation induces proliferation and migration of human keratinocytes enhancing wound re-epithelialization

Linda M van den Berg [a], Esther M. Zijlstra-Willems [a], Cornelia D. Richters [b,e], Magda M.W. Ulrich [c,d], Teunis B.H. Geijtenbeek [a,*]

[a] Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[b] Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands
[c] Department of Plastic, Reconstructive and Hand Surgery, VU University Medical Center, Amsterdam, The Netherlands
[d] Association of Dutch Burn Centres, Beverwijk, The Netherlands
[e] Euro Skin Bank, Beverwijk, The Netherlands

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ABSTRACT

Beta-glucans in temporary wound dressings have immuno-stimulatory capacities and have been shown to enhance wound healing in burn patients. Curdlan is a 1,3-linked bacterial/fungal derived beta-glucan that induces inflammatory responses via the C-type lectin receptor dectin-1 on dendritic cells (DCs). Here we investigated the effect of beta-glucan curdlan and the role of dectin-1 expressed by keratinocytes (KCs) in wound healing. Curdlan enhanced migration, proliferation and wound closure of human KCs in a dectin-1 dependent manner, both *in vitro* and *ex vivo*. Our data suggest that curdlan induces human KC proliferation and migration and could therefore be used in creams to enhance wound healing.

1. Introduction

Beta-glucans are polysaccharides composed of the monosaccharide glucose, linked with beta-glycosidic bonds. Beta-glucans form long polymers that mainly have been used in bioartificial skins in combination with gelatine and collagen as temporary wound dressing [4,6]. Collagen matrices with beta-glucans have been shown to improve burn wound healing and reduce pain [6], however the molecular mechanisms underlying the effect of beta-glucans on wound healing are poorly described. Dermal fibroblasts react to beta-glucans by producing interleukin-6 (IL-6) and increased proliferation, which is beneficial for restoring the dermal extracellular matrix and wound healing [13,15]. In addition, beta-glucans induce cytokine production and the release of reactive oxygen species (ROS) by macrophages and dendritic cells [2,10,19], which is thought to be beneficial for neutrophil infiltration, angiogenesis and wound healing [4]. Re-epithelialization of the wounded area is achieved by keratinocyte (KC) migration and proliferation [20]. Therefore, we studied the effect of beta-glucans on KCs in wound healing. Curdlan is a linear 1,3-beta-glucan of bacterial and fungal origin that is insoluble in water but soluble in alkaline solutions [17]. Curdlan is recognized by the C-type lectin receptor (CLR) dectin-1, which is an important pathogen recognition receptor (PRR) on dendritic cells (DCs) inducing immune responses [1]. Dectin-1 activates the transcription factor NF-κB via the tyrosine kinase Syk, resulting in anti-fungal cytokine production in dendritic cells [10]. KCs express the CLR dectin-1 [5], and produce ROS upon dectin-1 and toll-like receptor-2 (TLR-2) costimulation by *Mycobacterium ulcerance* [16]. Here we show that KCs increased proliferation, migration and enhanced wound closure upon curdlan stimulation, which is dependent on dectin-1. We describe here that the 1,3-beta glucan curdlan induced both migration and proliferation in human KCs via dectin-1 and therefore could be used to develop treatments targeting dectin-1 on KCs to enhance and improve wound healing.

2. Material and methods

2.1. Antibodies and reagents

The following antibodies and reagents were used: anti-human dectin-1 (R&D systems); anti-human Syk-Y525-526P (Cell

* Corresponding author. Address: Department of Experimental Immunology, Academic Medical Center (AMC), Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Fax: +31 20 697 71 92.
E-mail address: t.b.geijtenbeek@amc.uva.nl (T.B.H. Geijtenbeek).

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Signaling); anti-human CD1a (Santa Cruz); anti-human KI67 (BD); anti-human TNF, biotinylated anti-human TNF, anti-human IL-8, biotinylated anti-human IL-8 (all Biosource); goat-anti-rabbit Alexa-488; goat-anti-mouse Alexa-488 and -546; BRDU and BRDU reagent kit (all Invitrogen); beta-1,3-glucan hydrate from *Alcaligenes faecalis* (Curdlan; Sigma Aldrich); haematoxylin (Mayer); Eosin Y (Sigma Aldrich); PBA buffer (PBS pH 7.4 supplemented with 0.5% BSA and 0.02% azide).

2.2. Human skin 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 cut into pieces of 1 cm^2. Skin was burned by using the *Human Ex vivo Adjustable Temperature regulating-Machine* as described before (HEAT-M; [21]). Human skin samples were cultured in the human *ex vivo* wound healing model as described before [3]. Culture medium and curdlan were refreshed twice a week. Skin grafts were embedded in Tissue-Tek (Ted Pella) and snap-frozen in liquid nitrogen directly after burning or after 24 h of culturing and were subsequently used for immuno-histochemical analysis. To isolate primary KCs, epidermis was enzymatically degraded by trypsin and DNAse I, and the single cell suspension was layered on a lymphoprep (ficoll; Axis-shield) gradient. The pellet contained KCs. KCs were maintained in Keratinocyte-SFM COMBO medium (Invitrogen). KCs were grown to 80% confluence and splitted 1:10 once a week. At least 4 independent donors were used per experiment.

2.3. Monocyte isolation and DC differentiation

Monocytes were isolated from buffycoats. Buffycoats were mixed with Hank's Balanced Salt Solution (HBSS) and 1500 I.U. heparin (Leo Pharmaceuticals) and peripheral blood mononuclear cells (PBMC) were isolated by a lymphoprep gradient step. Monocytes were isolated from the PBMCs by a Percoll (Amersham Biosciences) gradient step. Monocytes were 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. At least 3 independent donors were used per experiment.

2.4. Immuno-histochemical staining

5-μm human cryo sections were air-dried and fixed in acetone for 10 min. Sections were stained with haematoxylin and eosin. Or sections were stained as described before [21]. 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. At least 4 independent donors were used per experiment.

2.5. Flow cytometry

KCs were used between passage 2 and 4, were grown 30% confluent and were 48 h incubated with 10 μg/ml curdlan or vehicle. BRDU was added 1:100 for 24 h and cells were subsequently trypsinized and fixed in 70% ethanol. Cells were treated with 2 M HCl for 20 min and neutralized with 0.1 M sodium borate pH 8.5 for 2 min. Then samples were stained with anti-BRDU-PE (dilution 1:50; Invitrogen) at RT for 20 min. At least 4 independent donors were used.

Or KCs and DCs were preincubated for 20 min with 1 mM sodium vanadate to inhibit phosphatases and cells were subsequently stimulated for 15 min with curdlan (10 μg/ml). Then cells were fixed in 4% PFA and permeabilized in 90% ice-cold methanol for 30 min. Cells were incubated with anti-human Syk-Y525-526P for 60 min followed by anti-Rabbit-Alexa 488 (5 μg/ml) for 30 min. At least 3 independent donors were used.

Or freshly isolated KCs were fixed in 4% PFA and stained with anti-dectin-1 Ab for 30 min in PBA or PBS/BSA/0.1% saponin. Then KCs were stained with anti-Mouse Alexa 488 (5 μg/ml) for 30 min PBA or PBS/BSA/0.1% saponin. Matched isotype antibodies served as negative control. At least 3 independent donors were used.

2.6. Confocal scanning laser microscopy

Keratinocytes were confluently grown on coverslips. Cells were fixed in 4% PFA and permeabilized in PBS with 0.1% saponin/1% BSA, before cells were incubated with anti-dectin-1 antibodies for 60 min at room temperature. Then cells were incubated with anti-mouse Alexa 546 secondary antibodies for 30 min at room temperature. Finally, the slides were counterstained with Hoechst for 2 min. Between all incubation steps, cells were extensively washed with PBS (pH 7.4). Matched isotype antibodies served as negative control and all controls were essentially blank. Cells were analyzed by a Confocal Laser Scanning Microscope (Leica). At least 3 donors were used.

2.7. Scratch assay

Keratinocytes were confluently grown in 24 well plates. A scratch was applied and migration of KCs into the wounded area was measured after 0 and 24 h by microscopy. KCs were cultured in the presence of curdlan (10 μg/ml) and/or anti-dectin-1 blocking antibody (10 μg/ml). The closure of the scratch was calculated as follows: the open area at $t = 0$ h was considered 100%. The area (A) at $t = 24$ h was divided by the area on $t = 0$ and multiplied by 100%. $A(t = 24) / A(t = 0) * 100\%$. The area of the scratch was measured by Adobe AutoCAD software (trial). At least 5–8 independent donors were used.

2.8. Elisa

Immuno-sorbant plates (Nunc) were coated with anti-cytokine antibodies. Supernatant of KCs or DCs stimulated for 24 h Poly(I:C) (10 μg/ml) or curdlan (10 μg/ml) were incubated for 2 h at RT. Then plates were incubated with biotinylated anti-cytokine antibodies. Peroxidase-labeled streptavidin was used to detect the biotinylated Abs and absorbance was read at 450 nm. At least 4 independent donors were used. Immuno-sorbant plates (Nunc) were coated with anti-cytokine antibodies.

2.9. Statistical analysis

Data were analysed by the non-parametric Mann Withney U test by GraphPad Prism software. Data are represented as mean ± SD. Statistical significance of the data was set at $p < 0.05$, $^{*}p < 0.05$; $^{**}p < 0.01$.

3. Results

3.1. Human keratinocytes express dectin-1 but do not produce cytokines upon stimulation

Keratinocytes were freshly isolated from human skin. In order to investigate the effect of beta-glucans on KCs we first analyzed the expression of beta-glucan receptor dectin-1 on human KCs by flow cytometry. Dectin-1 was expressed on the cell surface as well as intracellular (Fig. 1a). KCs were grown on cover slips and dectin-1 expression was visualized by confocal laser scanning microscopy.