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Epidermal keratinocytes initiate wound healing and pro-inflammatory immune responses following percutaneous schistosome infection

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ABSTRACT

Keratinocytes constitute the majority of cells in the skin's epidermis, the first line of defence against percutaneous pathogens. Schistosome larvae (cercariae) actively penetrate the epidermis to establish infection, however the response of keratinocytes to invading cercariae has not been investigated. Here we address the hypothesis that cercariae activate epidermal keratinocytes to promote the development of a pro-inflammatory immune response in the skin. C57BL/6 mice were exposed to *Schistosoma mansoni* cercariae via each pinna and non-haematopoietic cells isolated from epidermal tissue were characterised for the presence of different keratinocyte sub-sets at 6, 24 and 96 h p.i. We identified an expansion of epidermal keratinocyte precursors ($CD45^-$, $CD326^-$, $CD34^+$) within 24 h of infection relative to naïve animals. Following infection, cells within the precursor population displayed a more differentiated phenotype ($\alpha 6 integrin^-$) than in uninfected skin. Parallel immunohistochemical analysis of pinnae cryosections showed that this expansion corresponded to an increase in the intensity of CD34 staining, specifically in the basal bulge region of hair follicles of infected mice, and a higher frequency of keratinocyte $Ki67^+$ nuclei in both the hair follicle and interfollicular epidermis. Expression of pro-inflammatory cytokine and stress-associated keratin 6b genes was also transiently upregulated in the epidermal tissue of infected mice. In vitro exposure of keratinocyte precursors isolated from neonatal mouse skin to excretory/secretory antigens released by penetrating cercariae elicited IL-1α and IL-1β production, supporting a role for keratinocyte precursors in initiating cutaneous inflammatory immune responses. Together, these observations indicate that *S. mansoni* cercariae and their excretory/secretory products act directly upon epidermal keratinocytes, which respond by initiating barrier repair and pro-inflammatory mechanisms similar to those observed in epidermal wound healing.

1. Introduction

Schistosomes are a major parasite of humans currently infecting over 230 million people world-wide (World Health Organization (WHO), 2012, http://www.who.int/mediacentre/factsheets/fs115/en/) and causing a debilitating chronic disease (schistosomiasis) responsible for an estimated 70 million disability-adjusted life years per annum (King et al., 2005; King and Dangerfield-Cha, 2008). The larval stage (cercariae) of the schistosome life-cycle is the first to interact with the host and actively invades the skin via secretion of excretory/secretory (E/S) antigens (Mountford and Trottein, 2004; Jenkins et al., 2005b; Paveley et al., 2009), including proteolytic enzymes and immunogenic glycans (McKerrow et al., 1985; Knudsen et al., 2005; Curwen et al., 2006; Harn et al., 2009; Paveley et al., 2011). Following initial invasion most *Schistosoma mansoni* cercariae mature into schistosomula and reside in murine skin for at least 2 days, during which time the epidermal basement membrane provides a temporary barrier to onward migration (Wheater and Wilson, 1979). Following arrival in the dermis, schistosomula seek a blood vessel in order to exit the skin via an intravascular route, migrate via the lungs and mature into the adult stage of their life cycle in the hepatic portal system (Wheater and Wilson, 1979; Jenkins et al., 2005b).

Schistosome cercariae are known to affect the function of dermal and epidermal antigen presenting cells (APCs) of the innate immune system (e.g. Langerhans cells (LCs) and dendritic cells (DCs) (Angeli et al., 2001; Kumkate et al., 2007; Cook et al.,

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2011)). However, the role of non-professional immune cells such as epidermal keratinocytes has not been investigated in the context of cutaneous schistosomiasis. Keratinocytes are of particular interest since they constitute the majority of cells in the skin's primary barrier against invading pathogens, the epidermis (Wood et al., 1992; Martin, 1997; Madison, 2003; Pivarcsi et al., 2003), and are likely to be the first cell type exposed to *Schistosoma* cercariae and their E/S antigens. In addition to providing a physical barrier to infection, keratinocytes mediate skin homeostasis (Blanpain and Fuchs, 2009; Nagao et al., 2012) and respond rapidly to mechanical insult via secreting soluble factors (e.g. cytokines and chemokines (Hong et al., 2001; Nagao et al., 2012)) and increasing their proliferative responses in order to restore damaged tissue (Trempus et al., 2003; Smithgall et al., 2008; Gause et al., 2013). Keratinocytes are also known to be a source of stress-associated cytokines produced in response to a number of other cutaneous pathogens (e.g. *Candida albicans* (Wollina et al., 2004), *Trichobilharzia* spp. (Ramaswamy et al., 1995b) and *Sarcoptes scabiei* (Mullins et al., 2009)). Observations in mechanical wounding models suggest that phenotypic diversity between epidermal keratinocytes may also influence the development of such responses (Jensen et al., 2008; Blanpain and Fuchs, 2009; Nagao et al., 2012; Plikus et al., 2012). For example, keratinocytes located in different epidermal niches, particularly within hair follicle structures, display distinct chemokine repertoires involved in recruitment of epidermal LC precursors (Nagao et al., 2012). Thus both the nature of keratinocyte responses and their location within the epidermis may contribute to the initiation of immune responses to invading cercariae at the site of infection.

Unlike the larvae of *Schistosoma* spp. that infect birds, which elicit dermatitis at their point of entry in mammalian skin (Kourilova et al., 2004), a single percutaneous exposure to *S. mansoni* cercariae does not result in an overt tissue lesion in murine infection models. However, repeated exposure to *S. mansoni* cercariae causes more tissue damage than a single exposure (Cook et al., 2011) and promotes both angiogenic responses, (i.e. formation of new blood vessels from existing vessels) also active during wound-healing (Aynsley, S.A. 2011. Exploring the dermal immune and angiogenic responses to *Schistosoma mansoni*. PhD thesis, University of York, UK.), and a CD4+ T helper (Th) 2 polarised immune response (Cook et al., 2011). Identification of commonality between the Th2-type immune responses to helminth parasites, including schistosomes (Cook et al., 2011), and those involved in tissue repair suggest that there is cross-talk between the two pathways in affected tissues (Gause et al., 2013). Epidermis-derived alarmins such as IL-33 and thymic stromal lymphopoietin (TSLP), and the innate pro-inflammatory mediators IL-1α and IL-1β are involved at an early stage in these pathways and constitute one route by which physically restricted keratinocytes in the epidermis may influence infiltrating APCs and dermal stroma (Maas-Szabowski et al., 2000; Smithgall et al., 2008; Ramalingam et al., 2009; Gause et al., 2013). Thus, changes in epidermal keratinocyte subtypes may have important consequences for the subsequent activation and conditioning of acquired immune responses to larval schistosomula and tissue damage caused by parasite migration.

In this study, we address the hypothesis that percutaneous invasion by *S. mansoni* cercariae elicits changes in epidermal keratinocyte populations per se, as well as influencing expression of pro-inflammatory cytokines and alarmins in whole epidermal tissue. Since keratinocytes are in close contact with epidermal leukocytes (e.g. LC and γδ T cells), we investigated whether cercarial E/S antigens directly affect keratinocyte production of the cytokines IL-1α, IL-1β and TSLP, using an in vitro model of basal keratinocyte precursors grown in the absence of other cell types (Caldelari et al., 2000; Lichti et al., 2008). We believe that our observations provide the first indication that schistosome cercariae and their E/S products act directly upon epidermal keratinocytes, which respond by initiating barrier repair and pro-inflammatory mechanisms.

2. Materials and methods

2.1. Ethics statement

All experimental procedures involving animals were conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 and were approved by the University of York, UK, Ethics Committee.

2.2. S. mansoni parasites and percutaneous infection

Anaesthetised 6–10 week old female C57BL/6 mice were exposed via each pinna to 200 *S. mansoni* cercariae (Puerto Rican strain) for 20 min to allow percutaneous infection to occur (detailed method reported elsewhere (Hogg et al., 2003a)). Infected mice were culled at 6, 24 and 96 h p.i. and compared with age- and sex-matched uninfected control (naïve) mice. Cercarial E/S material (also termed 0–3 h released proteins (0–3hRP)) was collected from live transforming cercariae and concentrated as previously described (Jenkins and Mountford, 2005). An equivalent volume of RPMI medium without parasite material but prepared in the same way (control RPMI (cRPMI)) was used as a negative control for E/S stimulation.

2.3. Isolation of epidermal and dermal cells

Pinnae were removed and their thickness quantified using a dial gauge micrometre (Mitutoyo, Japan). Freshly isolated pinnae were then split along the central cartilage and each half portion was floated, dermis side down, on DMEM supplemented with 1% heat inactivated FCS, 50 U/ml of penicillin, 50 μg/ml of streptomycin and 10 mM HEPES (all from Life Technologies, UK; as described previously (Hogg et al., 2003b)) with the addition of 0.4 Wünsch units/ml of Liberase TL enzyme cocktail (Roche, UK) and were incubated at 37 °C for 30 min. Enzymes were neutralised with DMEM/10% FCS/penicillin/streptomycin/HEPES and the epidermal sheet was separated from the dermis before the tissues were separately minced using sterile scissors. Suspensions of epidermal and dermal cells were passed through a cell strainer (BD Biosciences, UK) and resuspended in fresh media prior to enumeration and assessment of viability using Trypan blue dye (Life Technologies).

2.4. Flow cytometry

Cell suspensions were washed in cold PBS, centrifuged at 800*g*, resuspended in PBS/0.1% LIVE/DEAD Fixable Viability aqua dye (Invitrogen, UK), and then incubated on ice for 30 min. Cells were washed in fresh PBS/1% FCS and resuspended in 10 μl of goat serum to block non-specific antibody binding for 30 min on ice. Cell surface markers were labelled using cocktails of specific fluorophore-conjugated rat anti-mouse antibodies (eBioscience, UK; Supplementary Table S1) and incubated on ice for 30 min. Cells were finally washed and resuspended in PBS/1% FCS prior to analysis using a Dako Cytomation Flow cytometer (Dako UK Ltd, UK). In some instances, cells were fixed in PBS/1% paraformaldehyde (PFA). Flow cytometry experiments were analysed as proportion and frequency data collated from four independent experiments for naïve, 6 h and 24 h p.i. and three experiments for the 96 h time-point ($n = 3$ mice per time-point per experiment).