

THE NOVEL INTERACTIONS OF *NECATOR AMERICANUS* WITH THE INNATE IMMUNE SYSTEM

AND

THE DEVELOPMENT OF A 3D IMMUNOCOMPETENT MODEL OF HUMAN SKIN

BY

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ABSTRACT

Background.

Necatoriasis is a neglected tropical disease caused by the insidious parasite *Necator americanus*. This hookworm infects and reinfects approximately 500 million individuals worldwide, with a further 5.1 billion at high risk for acquiring the infection. Despite the high level of reinfection, no lasting immunological memory develops in the host. Albeit the profound health implications, chronicity and public health burden in developing countries, many aspects of human *Necator americanus* infection, particularly early events at the interface with the host immune system, are under researched. These figures and facts highlight the need for new research elucidating the molecular interactions between *Necator americanus* and the innate immune system. This will aid in the rational design of innovative and more efficient intervention strategies against hookworm infection, which is an essential measure for disease prevention.

Objectives.

In the context of Necatoriasis, this thesis studied the physical interaction between infective *Necator americanus* larvae (L_3) with human dendritic cells (DCs) and epidermal keratinocytes, investigating the biological consequences. In addition, the development of a platform consisting of human keratinocytes, fibroblast and DCs on a 3D scaffold was constructed as an *in vitro* model of human skin.

Results.

The present thesis provides new insights into early immunological events at the interface of DCs and *Necator americanus* larvae and could explain how L_3 affect immunity upon initial interaction with antigen presenting cells.

For the first time, the data presented illustrates the sequestration of human DCs onto the sheath of L_3 infective *Necator americanus* larvae, triggering the hookworm to exsheath. Intriguingly, the exposed cuticle of the larvae had negligible interaction with the free DCs. The findings also illustrate that the interaction between DCs and the larvae is mediated via a mandatory interaction with C-type lectin receptors, dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and mannose receptor (MR). Blocking of either receptors with antibodies resulted in an inhibition of DC sequestration and aggregate formation. This demonstrates the biological relevance of previously identified lectin binding molecules on the *Necator americanus* larvae (L_3) sheath in the context of interacting with DCs.

These findings allude to a disparity between the surface chemistry of the sheath and larvae that could explain their differential ability to interact with DCs. While the exact nature of differences in the surface properties of the larvae and sheath are yet to be characterised, this data clearly indicates the presence of distinct chemical signatures on the cuticle sheath that attract DCs. However, this not only induces exsheathing but also enables larvae migration without being recognised or challenged by antigen presenting cells. A potential escape mechanism through

which the larvae could bypass the immune cells, creating a possible site of 'temporary immune privilege'.

DCs incubated with viable axenic larvae exhibited an immature phenotype as evidenced by the low expression of the maturation markers CD80, CD83, CD86, CD40, and HLA-DR. Subsequently, the ability of DCs to acquire a mature phenotype in response to co-stimulation with lipopolysaccharide (LPS) in the presence of *Necator americanus* was assessed. These data show that DCs treated with the larvae will remain responsive to LPS stimulation. Additionally while the axenised larvae do not induce any cytokine production by DCs, they seem to suppress LPS induced cytokine expression, however these changes were not statistically significant (p value ≤ 0.3). Furthermore, the cell-free culture media from DCs, matured in the presence of LPS, had no visible effects on the larvae. Intriguingly, matured DCs in LPS-free culture media render the larvae non-viable through a lysing mechanism, alluding to a modified paracrine signalling response by mature immune cells in culture with the parasite.

Interestingly, in the presence of epidermal keratinocytes, ex-sheathing was not mandatory to enable larval burrowing. In fact, only a small number of the larvae sheaths were recoverable from the apical surface of the keratinocyte layer; indicating preferential ensheathed larval burrowing. The data also illustrated the novel behavioural strategies promoting host invasion by *Necator americanus* larvae, in the presence of epidermal keratinocytes. Larvae were notably slower to exsheath in culture with keratinocytes and exhibited no vigorous movements

as observed in DC cultures. This was thought to prevent early exsheathing, as the advantage of larvae maintaining their sheath during the initial stages of infection is in theory highly beneficial.

Finally an immunocompetent tri-culture was developed on 3D layered PET scaffolds, encompassing epidermal keratinocytes and dermal fibroblasts, interspersed with DCs cultured at air liquid interface. A functional barrier was optimised, following which immune cell migration within the tri-culture system was observed successfully.

Conclusion.

Collectively, the sequestration of DCs onto the larvae sheath, suppression of maturation and cytokine expression, provides a possible explanation for the lack of a lasting immune response. These data provide novel insights into early immunological events at the interface of DCs, epidermal keratinocytes and *Necator americanus* larvae, which could explain how L_3 evade immunity upon initial interaction with antigen presenting cells.

To my son Nasir; I dedicate not only this Thesis, but my entire life's work.

To my mother: Dr. Maryan Qasim. You are my safety net and anchor.

My secure foundation.

To my father: Abdul-Aziz Hassan. You are my unwavering source of strength.

To my sister: Dr. Faiza Hassan. You are my mentor and guide.

To my siblings:

Abbas, from you I have learned the self-discipline required to succeed.

Zahra, you have taught me the importance of gratitude.

Rawdah, you inspire me to be courageous.

Ahmed, you are my voice of reason.

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ABBREVIATIONS

Activation-Motif	**ITAM**
Activator protein 1	**AP-1**
Adult hookworm	**L_5**
Adherens junctions	**AJ**
Air liquid interface	**ALI**
Ancylostoma duodenale	***A. duodenale***
Antigen presenting cells	**APC**
Antibodies towards C-type lectin receptors	**α-CLR**
B cell lymphoma 3	**BCL-3**
Calcium	**Ca**
Carbohydrate recognition domain	**CRD**
Carbon dioxide	**CO^2**
Celsius	**C**
Centimetre	**cm**
Crohn's disease activity index	**CDAI**
C-type lectin-like receptor-1	**CLEC-1**
C-type lectin receptors	**CLRs**
Cyanine 5	**Cy5**
Cytotoxic T cell	**T_c**
Danger associated molecular patterns	**DAMPS**
DC-associated C-type lectin 1/2	**Dectin 1 or 2**
DC immunoreceptor	**DCIR**

DC lectin	**DLEC**
Death inducing signalling complex	**DISC**
Dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin	**DC-SIGN**
Dichloromethane	**DCM**
Disability adjusted life years	**DALYs**
Eggs per faecal gram	**EPFG**
Endoplasmic reticulum	**ER**
E-selectin ligand	**ESL-1**
Extracellular matrix	**ECM**
Fas associated protein with death domain	**FADD**
Fluorescein isothiocyanate	**FITC**
Gap junctions	**GJ**
G-force	**G**
Glutathione-S-transferases	**GST**
Granulocyte macrophage colony-stimulating factor	**GM-CSF**
IFN-regulatory factor 9	**IRF-9**
IκB kinase subunit-ε	**IKKε**
Immortalized human dermal fibroblasts	**BJ-6**
Immortalized human epidermal keratinocytes	**HaCat**
Immunoglobulins	**Ig**
Immunoreceptor tyrosine based activation motif	
Immunoreceptor tyrosine-based activation motif	**ITAM**
Infectious non-feeding filariform	**L_3**
Interferon	**IFN**

Interferon-regulatory factors	**IRF**
Interleukin	**IL**
International Unit	**IU**
Intracellular adhesion molecule	**ICAM**
Janus kinase 1	**JAK1**
Junctional complex	**JC**
Keratinocyte mannose binding receptor	**KcMR**
Connector enhancer of kinase suppressor Ras1	**CNKSR1**
Kinase suppressor of Ras	**KSR**
Kilovolt	**kV**
Langerhans cell	**LC**
Lipopolysaccharide	**LPS**
Lymphocyte specific protein 1	**LSP-1**
L_3 *Necator americanus* - *Ancylostoma* secreted protein 2	**Na-ASP-2**
Magnetic resonance imagining	**MRI**
Mannose receptors	**MR**
Mannose binding lectin	**MBL**
Major histocompatibility complex	**MHC**
MBL-associated serine proteases	**MASPs**
Microlitre	**µl**
Micrometre	**µm**
Milligram	**mg**
Millimolar	**mM**
Millilitre	**ml**

Millimetre	**mm**
Minutes	**min**
Mitogen-activated protein kinase-activated protein	**MAPKs**
Mitogen-activated protein kinase-activated protein kinase 2	**MAPKAP-MK2**
Monocyte derived dendritic cells	**DCs**
Multiple sclerosis	**MS**
Myeloid differentiation primary-response protein 88	**MYD88**
Nano gram	**ng**
N. americanus Aspartic Protease-1	**Na-APR-1 (M74)**
N. americanus Glutathione S-Transferase-1	**Na-GST-1**
Necator americanus	***N. americanus***
Neutrophil inhibitory factor	**NIF**
Newcastle's Disease Virus	**NDV**
Nuclear factor-κB	**NF-κB**
Nucleotide-binding oligomerization domain-like	**Nod-like**
Ohm's per centimetre squared	**Ω/cm^2**
Paraformaldehyde	**PAF**
Pathogen associated molecular patterns	**PAMPs**
Pattern recognition receptors	**PRRs**
Peripheral blood monocytes	**PBMC**
Phosphate buffered saline	**PBS**
Phosphatidylserine	**PS**
Phycoerythrin	**PE**
Poly(ethylene terephthalate)	**PET**

Polytetrafluoroethylene	**PTFE**
Polytetrafluoroethylene (PTFE) platform support	**ScaffHolder**
P-selectin glycoprotein 1 ligands	**PSGL-1**
Pre-adult hookworm	**L_4**
Propidium iodide	**PI**
Rhabditiform stage 1 larvae	**L_1**
Rhabditiform stage 2 larvae	**L_2**
Signal transducer and activator of transcription	**STAT-1**
Src-homology-3	**SH3**
Superoxide dismutase	**SOD**
TANK-binding kinase 1	**TBK1**
T cell receptor	**TCR**
T follicular helper cell	**T_{FH} cell**
T helper	**Th**
Tight junctions	**TJs**
TIR domain-containing adaptor protein inducing IFNβ	**TRIF**
Toll–IL-1-resistence	**TIR**
Toll like receptors	**TLRs**
Transepithelial electrical resistance	**TEER**
Transforming growth factor-β	**TGF-β**
Trifluoroacetic acid	**TFA**
Tumour necrosis factor alpha	**TNF-α**
Reactive oxygen species	**ROS**
RAF proto-oncogene serine/threonine-protein kinase	**RAF**

Ultraviolet	**UV**
Vaccine Institute Product Development Partnership	**Sabin PDP**
Vascular cell adhesion molecule	**VCAM**
Volume per volume percent	**v/v %**
World Health Organization	**WHO**
Weight per volume percent	**w/v %**
Zonula occludens	**ZO-1**

CHAPTER 1: Introduction

1.1 General introduction

Among the parasitic nematodes that cause infection, *Ancylostoma duodenale* (*A. duodenale*) and *Necator americanus* (*N. americanus*) are the two major genera that account for the majority of human hookworm infection (Brooker, Bethony et al. 2004). The latter, however is more widely studied and has a higher prominent occurrence (Loukas, Hotez et al. 2016). The prevalence of *N. americanus* infection is generally highest in areas of underdevelopment and rural poverty (Hotez, Bethony et al. 2005), with the majority of incidences in the sub Saharan districts of Africa (Hotez and Kamath 2009) (Figure 1). A collaborative systematic analysis of the global burden of Necatoriasis was conducted in 2013, identifying approximately 500 million individuals affected worldwide (Vos T, Barber RM et al. 2015) particularly in countries with poor public health provisions. An estimated 80 million of infected individuals are classified under severe cases, resulting in a loss of approximately 22 million disability adjusted life years (DALYs) annually (Hotez, Bethony et al. 2006); with a further worldwide 5.1 billion at high risk for acquiring the infection (Pullan and Brooker 2012).

Despite its profound health implications, chronicity and significant public health burden in developing countries, many aspects of human *N. americanus* infection are under researched. Particularly early events at the interface with the host immune system, as the majority of research on Necatoriasis, has focused on the mature stages of the hookworm (Loukas, Hotez et al. 2016).

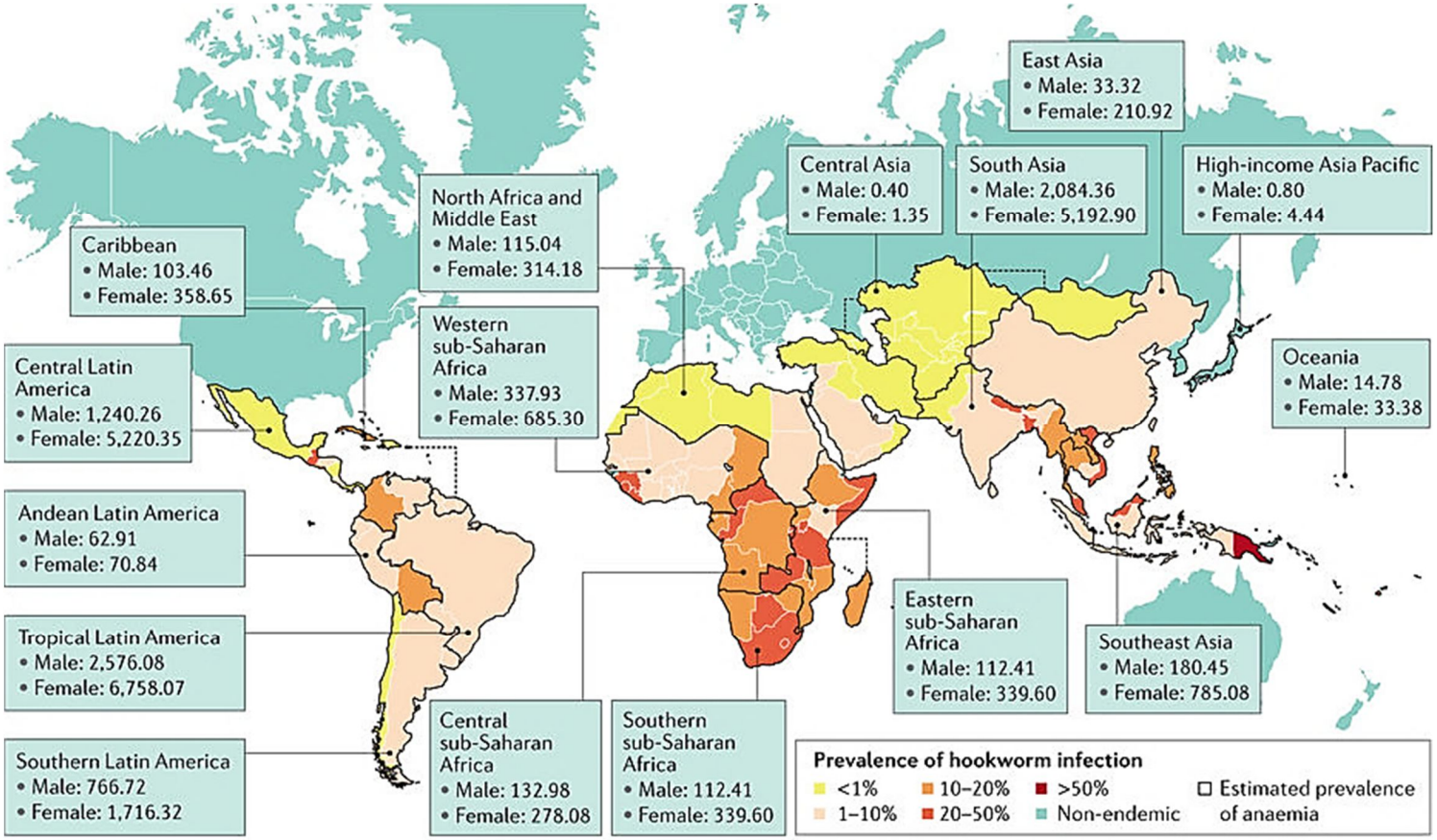


Figure 1: The prevalence of *Necator americanus*.

(Loukas, Hotez et al. 2016)

These insidious parasites, infect and reinfect (Loukas and Prociv 2001), following which no lasting immunological memory develops in the host (Geiger, Caldas et al. 2007). However whilst a few aspects of immunological responses to hookworms have been studied, they largely remain unknown. Efforts in developing novel therapeutic approaches could be helped by an increase in knowledge on the initial interactions between the *N. americanus* hookworm larvae and key components of the innate immune system. These include dendritic cells (DCs) and epidermal keratinocytes; exploring how such interactions influence downstream immunological events.

One of the major concepts within this thesis was to elucidate the biological relevance of the heavily glycosylated *N. americanus* larvae sheath. Early literature has identified a range of lectins isolated from plants capable of binding to sugars present on the L_3 sheath, including mannose, fucose, heparin sulphate and galactose (Kumar and Pritchard 1992). In the context of *N. americanus* life cycle, it has many opportunities to interact with the host DCs during the initial stages of infection (Quinnell, Pritchard et al. 2004, Geiger, Caldas et al. 2007). DCs are antigen presenting cells necessary for generating the adaptive arm of the immune system (Savina and Amigorena 2007). These sentinel cells express different types of pattern recognition receptors (PRRs) on their surface which bind specific pathogen associated molecular patterns (PAMPs). As part of their armoury of PRRs DCs express a range of C-type lectin receptors (CLRs) with a specificity for the recognition of glycosylated antigens (Thompson, Kaminski et al. 2011).

However, the biological relevance of the glycosylated *N. americanus* sheath in the context of interaction with CLRs on DCs has not yet been studied. Additionally, the *N. americanus* is thought to favour ensheathed larval burrowing, during the initial stages of infection (Kumar and Pritchard 1992). Nevertheless, there is no literature available on the purpose and immunological advantage of the larva sheath; a key factor which is investigated in this thesis.

Overall, the public health burden human Necatoriasis poses on the developing world highlights the need for new research to pave the way for the rational design of an innovative and more efficient intervention strategy against hookworm infection.

1.1.1 Clinical manifestation and life cycle of *Necator americanus*

This globally wide spread nematode, is an obligate skin penetrating parasite, causing severe gastrointestinal infections in mammals, and has been a major factor in the cause of anaemia and iron deficiencies in developing countries (Brooker, Bethony et al. 2004, WHO 2008, Hyun, Kim et al. 2010). Other symptoms associated with these voracious blood feeders include intestinal blood loss, protein deficiencies, emaciation, growth retardation, distended abdomen, cognitive impairment and in extreme cases cardiac failure (Albonico, Crompton et al. 1999, Devaney 2005, Bogitsh, Carter et al. 2012). Nonetheless the severities of these symptoms vary as it is dependent upon the intensity of the

infection, which more than often is moderate to severe (WHO 2002). The intensity of the infection is estimated and measured via a faecal hookworm egg count due to the nature of the *N. americanus* life cycle; the hookworm does not replicate within the human host (Schad, Chowdhury et al. 1973). The World Health Organization (WHO) has outlined that a faecal egg count exceeding 4000 eggs per faecal gram (EPFG) within an individual causes a high intensity of the infection that can result in mortality. However, EPFG's of approximately 2000 to less than 4000 are considered moderate (WHO 2002, Hotez, Bethony et al. 2005). Infection with *N. americanus* hookworm is normally asymptomatic but described to be silent and insidious, due to the chronic complications involved (Stoll 1962).

The journey of this parasite initiates following the pairing of adult worms in the jejunum and duodenum, occurring approximately 6-8 weeks subsequent to infection with the infective L_3 larvae. The hookworm reach sexual maturity and couple in the intestine, following which the female hookworm produces thin, hyaline ovoid unembryonated eggs (65-75 µm x 36-40 µm) (Hoagland and Schad 1978, Jian, Sen et al. 2003, Bethony, Brooker et al. 2006). The average life span of the *N. americanus* hookworm ranges between 3-5 years, and each gravid female worm can produce up to ten thousand eggs daily (Hoagland and Schad 1978). These are excreted in the soil and hatch after 1-2 days under moist conditions and temperatures of 23-30 ^{0}C (Schad, Chowdhury et al. 1973). The eggs then undergo cellular divisions and are embryonated (Pritchard, Quinnell et al. 1990, Loukas and Prociv 2001). Once hatched, the primary infantile form

is identified as a rhabditiform or stage 1 larva (L_1), which is characterized by a muscular oesophagus that has a flask like appearance. The larvae continue to grow, absorbing faecal microflora and organic debris before eventually shedding twice, reaching the stages rhabditiform (L_2) and the infectious non-feeding filariform (L_3) (Pritchard, Quinnell et al. 1990, Bethony, Brooker et al. 2006). The L_3 is covered by a thin sheath consisting of cuticles from the L_2 stage (Loukas and Prociv 2001, Brooker, Bethony et al. 2004). There are several evolving modifications to the L_3 as it is elongated up to 300-400 μm, the feeding has ceased and its progress to develop is halted. More importantly, the larvae is infectious at this stage (Loukas and Prociv 2001, Hotez, Bethony et al. 2005).

In order to initiate an infection, the L_3 larvae respond to specific stimuli such as the heat emitted by the target host, which signals a hookworm invasion strategy (Schad, Chowdhury et al. 1973, Pritchard 1995). The L_3 larvae arrange themselves via a process referred to as searching and waving, where the larvae body become erected and their anterior ends moves in multiple ways (Haas, Haberl et al. 2004, Haas, Haberl et al. 2005). This behaviour is generally referred to as 'questing' and significantly increases the parasites chances of finding a suitable host (Pritchard 1995, Hotez , Brooker et al. 2004). While the skin is anatomically an effective barrier against a wide range of pathogens, the L_3 larvae burrow mechanically into it by pressing their anterior against the skin and pushing into the surface (Pritchard 1995, Haas, Haberl et al. 2005). The process of mechanical burrowing is estimated to occur within approximately 5-10 minutes but this can take up to 2 hours subsequent to exposure (Matthews 1982).

Upon contact with the target host, this skin burrowing parasite interacts with possible lipid receptors and other molecules found on the skin surface, prompting the release of enzymatic secretions which digest skin macromolecules (Loukas and Prociv 2001).

Previous *in vitro* studies have observed the L_3 larvae to penetrate the epidermal and dermal layers of human skin, with a distinct passage of cellular destruction (Matthews 1982). However no such studies have been examined in detail, with a focus on the immunological advantage of exsheathing to the larvae. The ensheathed L_3 larva sheds its larval coat during the stages of skin penetration, although this is not always essential for causing infection, as murine studies have shown (Kumar and Pritchard 1992). Studies in *in vivo* reported the larvae capable of penetrating the skin without exsheathing, with only a few larval sheaths recovered on the outer skin surface following infection (Kumar and Pritchard 1992). Intriguingly, studies conducted in endemic areas of Papua New Guinea, reported that antibodies from infected individuals were only capable of recognising the antigens on the surface of the larvae sheath (Pritchard, Quinnell et al. 1990). In addition, following skin penetration, the larvae remain dormant (within the skin) for an estimated period of 48-72 hours post infection, as shown in neonatally infected hamster models (Behnke, Paul et al. 1986). This suggests that the L_3 larvae could possibly be diverting the host immune responses via a period of inactivity, and utilising the antigenic stimulation of its sheaths as an immune diversion. Other theories surrounding this phase include that the L_3 larvae require this time to activate developmental changes, as mice studies

reported the reversal of previously halted larval feeding (Hawdon, Volk et al. 1993).

Nonetheless, the activated L_3 larvae employ a range of secreted enzymes to continue and navigate their journey through the vasculature of the skin, via the epidermal and dermal layers. The larvae then ultimately enter the blood circulation through the capillaries (Hewitson, Grainger et al. 2009), where they are carried by the afferent circulation to the pulmonary vasculature and into the lung capillaries. The larvae then rupture the parenchyma and enter the alveolar spaces i.e., bronchioles, followed by migration into the trachea, where the larvae are coughed up and swallowed (Hotez , Brooker et al. 2004). At this stage the anterior of the hookworm begins to round and develops a pigment in its intestine. These are markers for developmental modifications, characterizing the lung mediated stage of Necatoriasis (Hoagland and Schad 1978, Jian, Sen et al. 2003). Subsequent to swallowing, the larvae reach the small intestine where further developmental changes occur and the larvae moult, maturing into a pre-adult hookworm (L_4) (Hawdon and Schad 1990).

In the intestine the L_4 larvae reach final maturity (L_5), which can be defined by the development of specific characteristics. These include the formation of a complete buccal capsule and dorsal/ventricular cutting plates found on the anterior and posterior of the hookworm. The adult hookworms are cylindrical in shape with the length varying in males (7-9 mm) and females (9-13 mm) (Pritchard, Quinnell et al. 1990, Jian, Sen et al. 2003, Hotez , Brooker et al.

2004). These modifications prompt the mature hookworms to hook/attach into the intestinal epithelium. Notably, feeding only initiates shortly before the production of eggs, and continuous incessantly thereafter with an estimated blood loss of 40 µl/worm daily (Hoagland and Schad 1978, Devaney 2005). Circulating back to the initial stages of the hookworm life cycle, where the female hookworms lays eggs that are shed in the host faecal excrements (Pritchard, Quinnell et al. 1990, Loukas and Prociv 2001). The complete hookworm life cycle and mature hookworm characteristics are depicted in Figures 2 and 3 respectively.

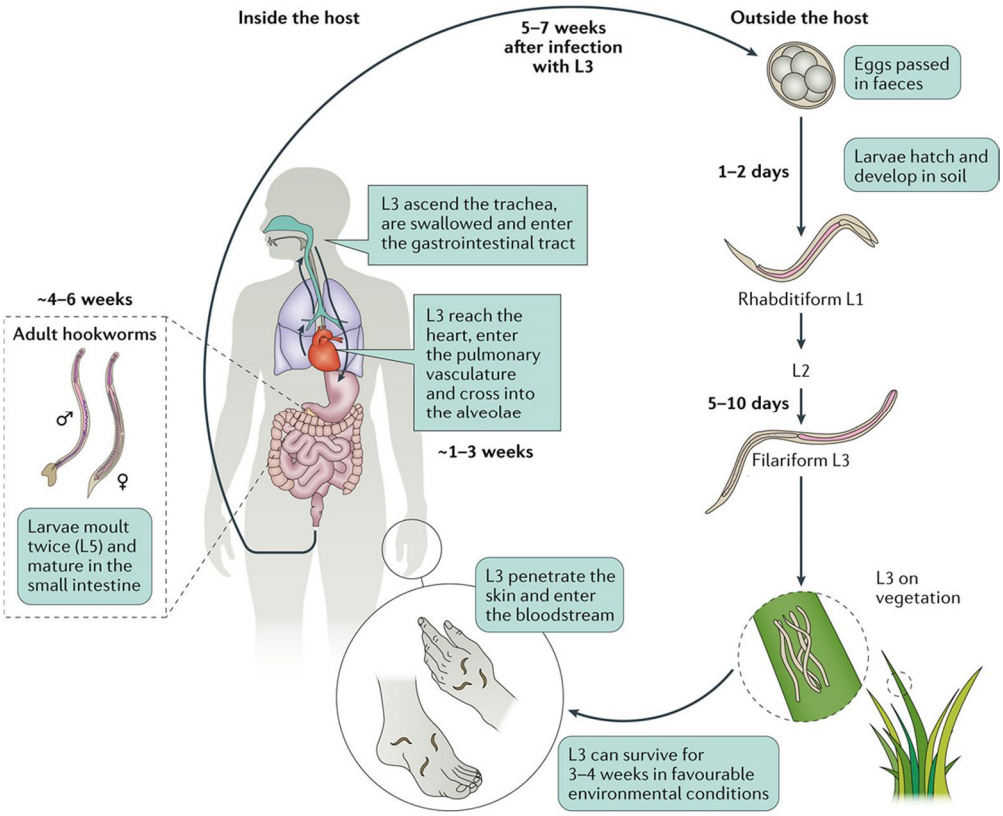


Figure 2: *Necator americanus* life cycle.

(1) Unembryonated eggs are excreted in faeces of infected individual. (2) Eggs hatch following 1-2 days in the faecal matter/soil to form the L_1 rhabditiform

larvae. (3) Larvae continue growing/feeding for 5-10 days in the faecal matter/soil, developing into the infective non-feeding L_3 larvae. (4) Upon contact, the L_3 larvae burrow mechanically into the skin, where they travel through the vasculature to the lungs. Here they further penetrate into the pulmonary alveoli, migrating towards the bronchial tree where they are finally swallowed down in the pharynx. (5) Upon reaching the small intestine, the larvae continue to develop into mature hookworms, anchoring into the epithelium and feed incessantly on intestinal mucosa/blood (Loukas, Hotez et al. 2016).

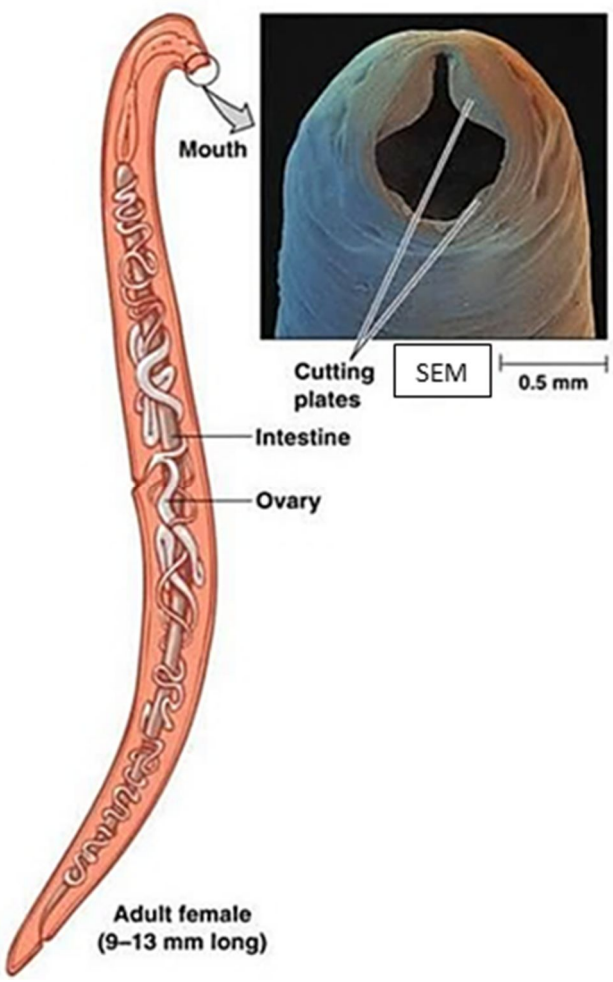


Figure 3: Scanning electron micrograph and schematic diagram illustrating the adult *Necator americanus* hookworm.

The mature hookworm has a buccal capsule which contains 2 dorsal and ventricular cutting plates as well as 2 sub-dorsal and ventral teeth situated towards the posterior of the nematode. The *N. americanus* is cylindrical in shape with the length varying in adult males (7-9 mm) and females (9-13 mm). These

features enable effective feeding on the intestinal mucosa and blood. Adapted from (Hotez, Bethony et al. 2005).

In this thesis the infective *N. americanus* (L_3) larvae were studied. Considering the *N. americanus* life cycle, once the non-feeding L_3 stage is reached these cannot revert back into their L_2 form. Additionally, development into the mature hookworm is stimulated by the L_3 larvae infecting the intestinal lumen and re-initiating feeding among other physical developmental changes (Hawdon, Volk et al. 1993). Identification of this species from others closely related parasites are via hallmark characteristics, including a non-notched pointed tail, the average measurements of 10 larvae ranging from 300-400 µm, and a clustering (bulb) of the oesophagus located at 1/3 along the hookworm's length (Pasuralertsakul and Ngrenngarmlert 2006, Loukas, Hotez et al. 2016). This ensures the larvae examined in this thesis are the infective, ensheathed L_3 *N. americanus* (Figure 4).

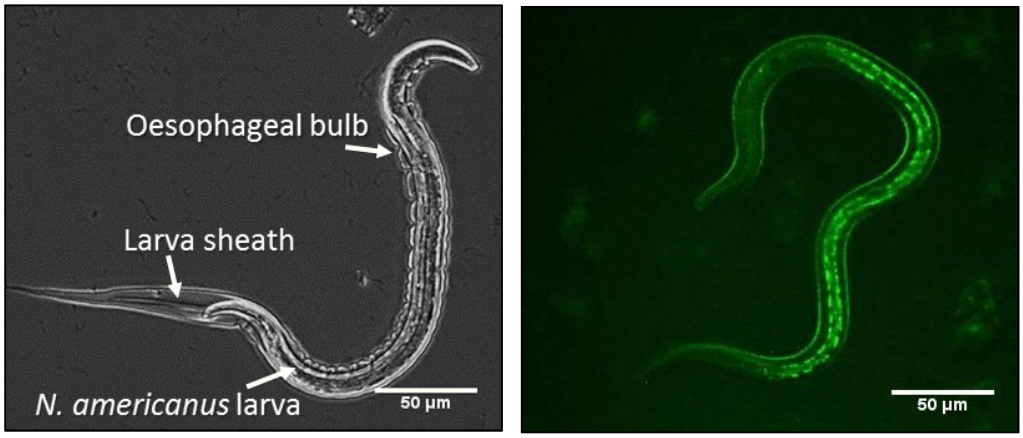


Figure 4: Illustrating the hallmark characteristic of L_3 infective *Necator americanus* larva identification (ZOE™ Fluorescent Cell Imager).

The L_3 ranges from 300-400 μm, with a pointed tail and a clustering (bulb) of the oesophagus located at a third along the hookworm's length. Images are representative of 3 larvae batches (n=3)

1.1.2 Immune response to *Necator americanus*

There have been several studies attempting to investigate human *N. americanus* hookworm infection in humans (Fujiwara, Geiger et al. 2006, Teixeira-Carvalho, Fujiwara et al. 2008, Fujiwara, Cancado et al. 2009). Nevertheless, due to the chronic symptoms associated with Necatoriasis, the application of chemotherapeutic drugs is obligatory (Holden-Dye and Walker 2007), which limits the research, providing only an immunological snapshot. To date there are a few publications which have employed low-dosage (25-50 larvae) controlled *in vivo* volunteer studies (Ogilvie, Bartlett et al. 1978, Maxwell, Hussain et al. 1987, Mortimer, Brown et al. 2006, Feary, Venn et al. 2010), which are generally unrepresentative as the majority of individuals in endemic areas have an infestation of over 100 larvae that can result in mortality if left untreated (Hotez , Brooker et al. 2004).

Nonetheless, there have been many attempts to establish a disease animal model for *N. americanus* hookworm infection (Fujiwara, Geiger et al. 2006). This research is driven by the demand for novel anthelminthic drugs and vaccine development, as the hookworm poses a great health burden in developing countries with an increasing resistance to available drugs (Geerts and Gryseels 2000, Holden-Dye and Walker 2007). There is a lack of an effective

experimental animal model since the majority develop atypical traits in comparison to a human hookworm infection. Animals which have been infected include chickens (Goyal and Johri 1986), rabbits (Bhopale and Menon 1979), chimpanzees (Miller 1968), mice (Girod, Brown et al. 2003) and hamsters (Jian, Sen et al. 2003). In mice the infective *N. americanus* does not develop into a blood feeding mature hookworm, resulting in a model that lacks the crucial components required for replicating both the symptoms and disease characteristics associated with human Necatoriasis. In canine and hamster studies, albeit the successful initial infection, the animals develop a natural resistance (Fujiwara, Geiger et al. 2006). This renders their biological relevance unrepresentative to the human disease particularly with regards to skin penetration and interaction with fundamental innate immune cells such as DCs within the skin (Miranda de Carvalho, Bonnefont-Rebeix et al. 2006).

DCs are potent antigen presenting cells (APC) necessary for generating the adaptive arm of the immune system, through the uptake of pathogenic antigens to prime T cells, effectively activating the adaptive responses (Iwasaki and Medzhitov 2015). Currently there is no extensive knowledge on the influence of *N. americanus* larvae on the function of DCs. However an immunoepidemiological study found that monocyte derived DCs from infected volunteers (as opposed to non-infected) had a down regulated expression of CD16, 86, CD1a, HLA-A, B, C and HLA-DR following stimulation with lipopolysaccharide (LPS) from *Salmonella* (Fujiwara, Cancado et al. 2009). The study found the DCs to be in a state of impairment, where the immune cells are

incapable of responding to a potent stimulant (i.e., LPS). This was also thought to be the cause for a decrease in T cell proliferative responses observed in human infection (Fujiwara, Cancado et al. 2009). Furthermore, human studies have shown the hookworm to elicit a mixed T helper (Th) 1 and 2 response characterized by a combined cytokine expression of interferon (IFN)-γ, interleukin (IL) - 4, 5, 12 and 13 (Pit, Polderman et al. 2001, Geiger, Massara et al. 2004, Quinnell, Pritchard et al. 2004). In addition there is an increase in $CD4^+$ memory cells (Kalinkovich, Weisman et al. 1998, Borkow, Leng et al. 2000) and a decrease in $CD3^+/CD4^+$ naive T cells (Onyemelukwe and Musa 2001). Although the percentages of T cells reported in infected humans were decreased, they were in a state of immune activation with significant increases in $CD8^+$, $CD4^+$, $CD3^+$/HLA-DR^+ T cells (Kalinkovich, Weisman et al. 1998, Borkow, Leng et al. 2000). Human studies have also observed an increase in antigen specific immunoglobulins (Ig), which are dependent on the stage as well as intensity of infection. For instance IgG and IgE increased in correlation with the intensity of the infection. In contrast, IgA is decreased as the intensity of infection increases; this is thought to be caused by the proteolytic activity of *N. americanus* secretions (Bentwich, Weisman et al. 1996, Loukas and Prociv 2001).

Continuing, Th2 responses are thought to be protective against *N. americanus* infection, as an increased level of IL-5 resulted in a decreased parasitic burden (Quinnell, Pritchard et al. 2004). This concurs with evidence from animal models where localised Th2 responses are important for conferring protective immunity

(Girod, Brown et al. 2003, Fujiwara, Loukas et al. 2006), however the precise mechanisms are poorly understood. Moreover, Th2 cells also activate B cells with IgE antibodies (Pritchard, Quinnell et al. 1995) and secrete IL-13 (Wright and Bickle 2005), stimulating intestinal mucosal mast cells to release histamine. This induces peristalsis and the acidification of gastric fluid, aiding in the expulsion of helminths from the body (McKean and Pritchard 1989, Zaph, Cooper et al. 2014). Other characteristics of Necatoriasis include eosinophilia (Bentwich, Weisman et al. 1996, Loukas and Prociv 2001), which was further corroborated in a self-infliction study, observing an upregulation in eosinophil count 72 hours post the initial infection (Wright and Bickle 2005). It is thought that host eosinophil's in combination with the complement system bind to the larval form of the hookworm, due to the predominant and significant increase of these inflammatory mediators at the site of infection (Wright and Bickle 2005).

It is imperative to note that research on the exact immune response to the hookworm infection is still unclear due to the variable responses in different hosts.

1.1.3 *Necator americanus* and the hygiene hypothesis

The hygiene hypothesis is based on the premise that the occurrence of allergic diseases are predominant in urban white communities where the economy is highly developed. This was in comparison to rural communities, where the

occurrence of helminths, bacterial and viral infections are substantially higher (Gerrard, Geddes et al. 1976). This concept has since generated an interest in the possibility of an anti-inflammatory hookworm therapy, fuelling research into many auto-immune diseases and inflammatory conditions; intriguingly in line with the hygiene hypothesis (Nicolaou, Siddique et al. 2005, Asher, Montefort et al. 2006).

The immune suppressive properties of *N. americanus* was investigated in 32 asthmatic adults for 16 weeks. This study reported a non-significant improvement in airway responsiveness following administration of 10 *N. americanus* larvae (Feary, Venn et al. 2010). In Crohn's disease, a small study in 9 patients infected with 25-50 larvae was observed for a period of 20 weeks, where a potential role for *N. americanus* immunomodulation was established. Out of the 9 volunteers, the Crohn's disease activity index (CDAI) for 7 patients was significantly improved; however 2 experienced an increase in CDAI (Croese, O'Neil et al. 2006). Furthermore, the effects of the infectious parasite was also studied in coeliac disease, with a specific allergy towards gluten. In this study, a cohort of 20 patients were treated with 5-10 larvae for 20 weeks following which no significant improvements were observed in response to a wheat challenge (Daveson, Jones et al. 2011). Additionally, a multiple sclerosis (MS) study in 24 volunteers compared the effects of helminths on disease progression over a period 4.5 years (Correale and Farez 2007). The patients, which were infected with a variety of helminths, had significantly fewer relapses and a decreased magnetic resonance imagining (MRI) activity. Interestingly,

following the administration of anti-helminthic drugs, the primary improvements observed in these patients deteriorated. This was thought to be caused by the decrease in IL-10 and transforming growth factor-β (TGF-β), combined with an increase in INF-γ and IL-12. This data alludes to a possible therapeutic benefit of helminths in the treatment of MS patients (Correale and Farez 2007). These findings were employed as a basis for *N. americanus* clinical trials at the University of Nottingham, which to date is the first trial administering a controlled dose of *N. americanus* for the treatment of MS (Tanasescu and Constantinescu 2015).

Epidemiological studies have observed a lower frequency of MS and other autoimmune or allergic diseases in helminth carriers (Nicolaou, Siddique et al. 2005, Asher, Montefort et al. 2006), with studies in murine models reporting protective immunity in such diseases. These include type 1 diabetes (Zaccone and Hall 2012), MS (La Flamme, Ruddenklau et al. 2003, Walsh, Brady et al. 2009), asthma (Kitagaki, Businga et al. 2006) and extreme allergic responses in anaphylaxis (Mangan, Fallon et al. 2004). Nonetheless it is imperative to note that the larvae dose employed in these animal studies are grossly disproportionate to the human hookworm burden. The majority of animal studies have also proposed a preventative role for helminths, with only a few reporting helminths to actually impact disease progression (Helmby 2009). Notably, there is a possibility that the lack of allergic/autoimmune diseases observed in endemic areas of Necatoriasis or general helminth infections could be in line with these findings. This would imply that the parasite offers a ‘preventative protective

immunity' as opposed to a therapeutic treatment for established diseases (Helmby 2015).

Infection with *N. americanus* is associated with a variety of symptoms; initially a mild clinical inflammation where the larvae penetrates the skin accompanied with redness, itching and migratory creeping (Loukas, Hotez et al. 2016). In the lungs the larvae result in inflammatory lesions, and once in the intestine causing nausea and abdominal pain along with the previously discussed pathological symptoms (Hotez , Brooker et al. 2004, Wright and Bickle 2005). Due to the stages involved in the *N. americanus* life cycle, it has many opportunities to encounter and interact with the host barrier tissues. Subsequently, presenting the immune system with a variety of hookworm antigens and secretions which occur at different stages throughout the course of infection (Geiger, Caldas et al. 2007). Despite such diverse antigenic stimulation, no lasting immunity develops and whilst some aspects of immunological responses to hookworms have been studied, the exact functional mechanisms remain unknown (McSorley, Hewitson et al. 2013).

1.1.4 *Necator americanus* secreted products

There are a variety of secretions by the *N. americanus* hookworm which facilitate the process of infection. It is believed that the secretions enable the hookworm to modulate the host immune response, as many studies have shown

various hookworm secretions are involved in causing a down regulation of the immune system (Brown, Girod et al. 1999, Loukas and Prociv 2001, Wright and Bickle 2005, Teixeira-Carvalho, Fujiwara et al. 2008, Hewitson, Grainger et al. 2009).

The *N. americanus* are thought to protect vulnerable and exposed exteriors (i.e., the inner larval surface) via the secretion of functional antioxidants as a defensive neutralizing agent. This is to counter the effects of leukocyte oxidative bursts which produce free radicals, including superoxide dismutase (SOD) and glutathione-S-transferases (GST), a lipid hydroperoxide (White, Maxwell et al. 1986, Brophy, Patterson et al. 1995). In addition, research has found that the vaccine selected candidate, L_3 *Necator americanus* - *Ancylostoma* secreted protein 2 (Na-ASP-2), functions as a chemoattractant via molecular mimicry of cc-chemokines (Bower, Constant et al. 2008). This protein has been shown to significantly recruit neutrophils and monocytes to the site of infection in both *in vivo* and *in vitro* conditions. It is thought that the inflammatory response will increase tissue permeability and oedema, thus enabling a swifter infiltration of the larvae into the skin (Bower, Constant et al. 2008).

Furthermore, another theory is that the recruitment of neutrophils and monocytes will guard the hookworm larvae against more potent immune cells. This would ensure the prevention of natural killer cells binding to the antibody coated parasite, via the CD16 receptor and releasing IFN- γ. Other immune responses which are also very potent include cytotoxicity caused by eosinophil dependent antibody responses, leading to the degranulation and release of eosinophil

derived granules resulting in tissue damage and dysfunction (Attallah, Lewis et al. 1980, Bower, Constant et al. 2008).

The group of CLRs receptors, found in the serum and on the surface of antigen presenting cells such as the dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on DCs and those of endothelial nature i.e., C-type lectin-like receptor-1 (CLEC-1), are fundamental components in the generation of an immune response against *N. americanus* (Engering, Geijtenbeek et al. 2002, Tanne and Neyrolles 2010, zquez-Mendoza, Carrero et al. 2013). The CLRs can dimerize to act as PRRs to recognise PAMPs, such as mannose or fucose components of bacterial secretions and hookworms (Janeway and Medzhitov 2002). Intriguingly, larvae derived CLRs that are analogous in both structure and function to the human form have been identified, which are thought to be secreted by the hookworm in order to compete with the host CLRs and avert the activation of immune cells (Loukas and Maizels 2000, Loukas, Brown et al. 2002). This immune evasive property is thought to promote incessant feeding in the intestine.

Moreover, although the functional properties remain to be determined, distant homologues of the neutrophil inhibitory factor (NIF) found in the secretions of adult *Ancylostoma caninum* have been reported in expressed sequence tags of *N. americanus* (Daub, Loukas et al. 2000). These NIFs are thought to inhibit components of the Complement Receptor 3 which are vital in cellular adhesion and fibrinogen interaction (Loukas and Prociv 2001). The inhibition of the CD11b and CD18 integrins not only prevents the production of cellular

hydrogen peroxide, but also inhibits the adhesion of activated neutrophils to the lining of the vascular endothelium in the gut (Moyle, Foster et al. 1994). This ensures the evasion of neutrophil recruitment to the site of feeding and thus evading the immune cells.

Other secreted products of the hookworm include acetyl cholinesterase (McLaren, Burt et al. 1974, Pritchard, Leggett et al. 1991), an immunogenic enzyme in Necatoriasis (Ogilvie, Bartlett et al. 1978). This is thought to be used for the hydrolyzation of gut derived acetylcholine and degradation of anti-coagulant proteins, assumed to inhibit host gut peristalsis and promote *N. americanus* feeding. This results in an effective delay of blood clotting in order to increase the volume of blood consumed by the larvae (Loukas and Prociv 2001).

The oesophageal glands found on the *N. americanus* hookworm are believed to contain secretory granules which store a vast range of proteolytic enzymes such as metallo- (Culley, Brown et al. 2000), cysteine (Ranjit, Zhan et al. 2008) and serine proteases (Kumar and Pritchard 1992, Kumar and Pritchard 1992, Hewitson, Grainger et al. 2009). These proteases have gelatinolytic properties and thus function to degrade components of the skin (Brown, Girod et al. 1999). Earlier studies have shown that larval secretions with aspartyl proteinase or metalloproteinase activity, could hydrolyse both elastin and collagen, whereas serine proteases were restricted to the hydrolyzation of elastin. Further *in vivo* hamster infection models, confirmed that the hydrolysing effects were inhabitable via pepstatin-A, which prevented larval burrowing. A factor which

establishes and highlights the importance of aspartyl proteinase secretions by the larvae, in order to penetrate the skin (Brown, Girod et al. 1999).

The metalloproteases in particular have been shown to down regulate eosinophil recruitment during inflammation by cleaving the crucial cytokine, eotaxin-1 (Culley, Brown et al. 2000). In addition, the cysteine proteases are thought to enhance IgE reactions, responsible for hyper sensitivity type 1 observed in infected patients. These individuals were found to have elevated levels of the low affinity Fcε receptor CD23, alluding to a possible linked mechanism (Pritchard, Kumar et al. 1993, Loukas and Prociv 2001). The aspartic proteins found in the hookworm secretions also aid in blood feeding by hydrolysing haemoglobin in the consumed erythrocytes (Williamson, Brindley et al. 2003).

Calreticulin, a multifunctional protein which is involved in molecular chaperoning, prevents the exportation of misfolded proteins from the endoplasmic reticulum. It also binds calcium involved in transcription regulation, integrin-mediated signalling and cell adhesion (Gold, Eggleton et al. 2010, Jiang, Dey et al. 2014). This protein has been purified from the secreted extracts of *N. americanus* and has been acknowledged as one of the core allergens identified by IgE in individuals infected with this host obligate nematode (Pritchard, Brown et al. 1999). Calreticulin is known to have comparable features and properties to lectin, and it is capable of binding components of the complement system (C1q) (Kasper, Brown et al. 2001). This *N. americanus* secretion is thought to influence the production of IgE, via the

promotion of cross linked surface glycoproteins and interaction with integrins (Reilly, Larkin et al. 2004).

1.1.5 Treatments for *Necator americanus* infection

There are numerous anthelminthic drugs available to treat hookworm infections. Nevertheless, the class of Benzimidazoles are the most widely used, covering an extensive family of chemicals, which treat nematode and trematode infections (Xiao, Hui-Ming et al. 2005, Wu, Fang et al. 2006, Holden-Dye and Walker 2007). There are currently four drugs which are employed in order to control the morbidity of helminth infections, these include levamisole, pyrantel pamoate, albendazole and mebendazole (WHO 2006). The primary two act as depolarizing neuromuscular blocking agents, causing the paralysis of the hookworm. In contrast the latter two function by preventing polymerization of microtubules within the hookworm, through the inhibition and binding of the beta-tubulins (Hu, Xiao et al. 2009, Martin and Robertson 2010, Aleyasin, Karuppagounder et al. 2015). These drugs are used against a wide spectrum of parasites, including round worms, tape worms and adult flukes. They have a large safety margin, where the drugs have a significantly higher affinity for the hookworm, in comparison to the host cells. Overall, these anthelminthic drugs are excreted swiftly and have a therapeutic dose which eliminates the helminths, with no binding to host tissues (Xiao, Hui-Ming et al. 2005, Wu, Fang et al. 2006, Holden-Dye and Walker 2007).

Recent systematic reviews have investigated the efficacies of these widely used drugs, in relation to a single dose treatment for hookworm infection. The results found that oral albendazole administration at a dose of 400 mg, had an efficacy cure rate of 66% (Cline, Little et al. 1984), whereas mebendazole had a cure rate of 31% following a dose of 500 mg (Albonico, Ramsan et al. 2002). Tribendimine administration at 400 mg resulted in 86% patient cure rates in clinical trials, which could identify this drug as an alternative future choice for treatments (Xiao, Hui-Ming et al. 2005, Wu, Fang et al. 2006). The benzimadoles are the most common drugs employed due to their high efficacy and low cost combination, although this increases the potential for hookworm drug resistance significantly (Xiao, Hui-Ming et al. 2005, Wu, Fang et al. 2006, Holden-Dye and Walker 2007).

Albeit the use of anthelmintic drugs is effective, it has proven to be difficult to implement in developing countries as there is a significant high level of rapid reinfection, rendering their usage futile (Loukas and Prociv 2001, Geiger, Caldas et al. 2007). Therefore, an alternative method such as effective vaccinations for the prevention of this disease is vital as this provides protection against the *N. americanus* hookworm larvae.

1.1.6 Vaccine development for *Necator americanus* infection

Necatoriasis and other common intestinal helminths are primarily associated with poor public health provisions and sanitation (Harhay, Horton et al. 2010, Mascarini-Serra 2011). However, addressing these issues alone through preventative measures such as protective foot wear (Tomczyk, Deribe et al. 2014) and an increase in personal sanitation, have shown no impact on the prevalence as well as intensity of infection. Additionally, as there is no accompanying health education programme, and a lack of economic development (Mascarini-Serra 2011). Generally, anthelminthic drugs are employed to control and treat *N. americanus* infection. Nevertheless, due to the high level of reinfection, it is necessary to develop a human hookworm vaccine which can provide host protection at the early stages of infection (Plotkin, Diemert et al. 2008, Bethony, Cole et al. 2011).

An effective vaccine must be comprised of different antigens, primarily derived from the penetrating larvae and the full-grown blood feeding adult worm. The combination of these antigens, is hypothesised to significantly enhance protective immunity, and improve or eliminate the pathology associated with the disease (Plotkin, Diemert et al. 2008, Hotez, Diemert et al. 2013). Founded on this basis, the proteins associated with the different stages of hookworm maturity have been identified as promising vaccine components. To date, the Sabin Vaccine Institute Product Development Partnership (Sabin PDP) is developing a bivalent human hookworm vaccine, formulated on an aluminium hydroxide

adjuvant (Alhydrogel®). This vaccine is comprised of *N. americanus* Glutathione S-Transferase-1 (Na-GST-1), and the *N. americanus* Aspartic Protease-1 (Na-APR-1 (M74)), and is currently in Stage 1 clinical trials (Sabin PDP 2017). The study is being conducted in 60 healthy adults residing in endemic areas in Brazil, where the safety of the vaccine and host immune responses generated are under investigation. This vaccine targets the chronically impairing symptoms associated with the later stages of infection and is intended for children of primary/school age (Sabin PDP 2017).

In matured adult hookworms, Na-GST has been identified as key antigen for vaccine development (Zhan, Liu et al. 2005, Hotez, Diemert et al. 2013, Asojo and Ceccarelli 2014). The Na-GST-1 is generally secreted by the hookworm to detoxify host derived free radicals. Studies in mice vaccinated with this antigen reported a mixture of T helper 1 and 2 responses. This was characterized by a strong antibody production of IgG1/2, as well as an increase in inflammatory mediators such as IFN- γ and IL-4. This is thought to prevent the action of the matured hookworm in the small intestine (Pearson, Pickering et al. 2010). The haemoglobinase, Na-APR-1 (M74), has also been investigated for vaccine development as it initiates the digestion of haemoglobin by the parasite, therefore targeting the later stages of the infection (Williamson, Brindley et al. 2003, Loukas, Bethony et al. 2005). Studies in dogs immunized with the antigen noted a substantial decrease in blood loss, with a reduction in both hookworm burden and faecal egg count (Loukas, Bethony et al. 2005).

Nevertheless, research into vaccines continues with a great focus into the differential stage specificity of antigens associated with human hookworm infection. In larval hookworms, these include the L_3 *Necator americanus* - *Ancylostoma* secreted protein 2 (Na-ASP-2), the multifunctional protein Calreticulin and metalloproteases (Williamson, Lecchi et al. 2004, Winter, Davies et al. 2005, Bower, Constant et al. 2008).

The Na-ASP-2 secreted proteins, have shown promising results, particularly in animal challenge infection studies, where the recombinant vaccine provides substantial protection. These proteins induce a protective immune antibody response in rats and reduce hookworm burden. In addition, the production of antibodies towards this antigen in individuals from endemic areas, correlates with a low risk for acquiring *N. americanus* infection. These findings led to the selection of Na-ASP-2 for further development in clinical trials, where this vaccine was found to induce generalized urticaria. This was due to the specific IgE production towards this antigen in *N. americanus* infected individuals, resulting in adverse allergic reactions following the administration of the recombinant vaccine and subsequent withdrawal of the vaccine trial. (Bower, Constant et al. 2008, Diemert, Pinto et al. 2012). Calreticulin has shown similar protective properties in vaccinated mice. A strong Th2 response was elicited, combined with a significant decrease in the worm burden observed in the lungs. Furthermore, immunizations with vaccines derived from irradiated *N. americanus* larvae exhibited a high immune response. Although this was not

protective, there was a reduction in the general hookworm pathology (Winter, Davies et al. 2005, Jian, Jun-Min et al. 2006).

Collectively, in order to appreciate the immune modulatory effects of the *N. americanus* hookworm, it is essential to review the immune system.

1.2 The immune system overview

In the context of human hookworm infection, previous literature has identified a range of lectins isolated from plants capable of binding to glycosylated molecules present on the L_3 sheath. These include mannose, fucose, heparin sulphate and galactose (Kumar and Pritchard 1992). In the context of the *N. americanus* life cycle, it has many opportunities to interact with the host DCs during the initial stages of infection (Quinnell, Pritchard et al. 2004, Geiger, Caldas et al. 2007). Albeit studies have shown the hookworm to elicit a mixed T helper 1 and 2 response in human hosts, there is currently no extensive knowledge on the influence of *N. americanus* larvae on the potent role of innate immune DCs (Loukas and Prociv 2001, Fujiwara, Cancado et al. 2009, Mendlovic and Flisser 2010). Previous studies have found that monocyte derived DCs from infected volunteers (as opposed to non-infected) had a down regulated expression of DC markers i.e., CD86, CD11c, following stimulation with LPS, alluding to parasite impaired DCs in human hosts (Fujiwara, Cancado et al. 2009).

DCs are master regulators of the immune system, and have a central role in the initiation of antigen-specific responses. Combining the microbial sensing features of the innate immune response, to the specific T cell and antibody responses of the adaptive system (Bonilla and Oettgen 2010, Turvey and Broide 2010, Iwasaki and Medzhitov 2015). Therefore, given that there is a lack of knowledge on DC interaction with *N. americanus*, the function of DCs in the innate and adaptive immune system are reviewed.

1.2.1 Innate inflammatory responses

Following the breach of the innate anatomical barrier, namely the skin in human hookworm infection, the inflammatory response is initiated. This comprises a cascade of events that serves to create an immediate barrier to pathogens and promote healing of subsequent tissue injury. This is characterised by the classical symptoms associated with both inflammation and *N. americanus* skin penetration; pain, swelling and redness (Feary, Venn et al. 2010, Liu and Cao 2016). During inflammation, immune cells present in the tissues and circulating blood, recognise PAMPs on invading pathogens, via PRRs on their surface (Mogensen 2009). Upon encounter, mast cells residing in the tissues de-granulate rapidly to release histamine, a cell derived inflammatory mediator, from storage granules and into the local microenvironment (Urb and Sheppard 2012). Histamine is a vasodilator and activates the endothelium, leading to an

increase in vascular permeability, triggering local oedema and depolarization of nerve endings. This accounts for the topical swelling, redness and itching observed (Urb and Sheppard 2012); an immune response which is thought to aid *N. americanus* larvae in penetrating the skin (Loukas and Prociv 2001). Furthermore, the sudden increase in vascular permeability enables immune cells such as neutrophils and monocytes to migrate into the inflamed tissue via diapedesis, where they can then function as macrophages (Muller 2011, Urb and Sheppard 2012) .

During this process, the activated tissue macrophages secrete a range of cytokines and chemokines. These include IL-1 and TNF-α, which prompts the endothelial cells of the blood vessels (at the site of infection) to begin expressing cellular adhesion molecules such as P-selectins (Nourshargh and Alon 2014). Factors which can induce the synthesis of P-selectins include the complement fragment C5a, leukotriene B4 and histamine, among others (Charles A Janeway, Paul Travers et al. 2001). Rolling adhesion is initiated as the P-selectins on endothelial cells, form transitory bonds with their corresponding P-selectin glycoprotein 1 ligands (PSGL-1). The rapidly moving leukocytes in the bloodstream slow down as a result of this interaction. E-selectins on endothelial cells, are synthesised following P-selectin expression; this binds E-selectin ligand 1 (ESL-1) on leukocytes (Charles A Janeway, Paul Travers et al. 2001, Nourshargh and Alon 2014).

The extracellular chemokines also trigger the release of intracellular pre-formed β2 integrins, primarily expressed on leukocytes. Other integrins such as β1 are also expressed on lymphocytes and monocytes. These migrate to the cell surface, binding endothelial adhesion molecules such as intracellular adhesion molecule (ICAM)-1, 2 and vascular cell adhesion molecule (VCAM)-1 respectively (Faveeuw, Di Mauro et al. 2000). This tight adhesion arrests cell movement and enables transmigration to be initiated. Immune cells present in the circulation, move across the endothelial blood vessels and into the tissues where they function as macrophages (Nourshargh and Alon 2014). The *N. americanus* are thought to prevent or delay transmigration, as hookworm NIFs inhibit components of the Complement Receptor 3. This would prevent the adhesion of activated neutrophils to the lining of the vascular endothelium, which deters neutrophil recruitment to the sites of larval feeding or initial infection (Moyle, Foster et al. 1994, Loukas and Prociv 2001).

During phagocytosis, the immune cells, namely macrophages, neutrophils and DCs, phagocytose the invading pathogen via a cascade of events (Savina and Amigorena 2007). These are activated via the recognition of PAMPs or binding to opsonins such as complement component C3b and antibodies. The phagocyte then extends long actin-rich membrane projections, mediated by actin-myosin contractions (Tohyama and Yamamura 2006, Freeman and Grinstein 2014) and engulfs the pathogen. The phagosome formed then fuses with a lysosome in the cell, producing a new construct, the phagolysosomes (Savina and Amigorena

2007, Flannagan, Cosio et al. 2009). The pathogen is terminated within this intracellular vacuole (Flannagan, Cosio et al. 2009).

The *N. americanus* is identified as a highly adapted pathogen, with secretions that can cleave IgA, among other isotypes, from which Fab fragments are obtained. These in combination with NIFs are then employed by the larvae to block IgG or IgM mediated phagocyte attacks and complement components (Pritchard 1995).

1.2.2 Role of dendritic cells in the innate and adaptive immune system

Derived from the bone marrow, DCs are found circulating the blood, peripheral and lymphoid tissues, where they are ideally positioned to encounter antigens from the external environment. DCs are APC, necessary for generating the adaptive arm of the immune system (Stockwin, McGonagle et al. 2000, Howard, Charleston et al. 2004). These sentinel cells express different types of PRRs on their surface that bind specific PAMPs, which are conserved features of pathogens, i.e., LPS component found in all gram-negative bacteria (Silhavy, Kahne et al. 2010). The PRRs expressed on DCs include Toll like receptors (TLRs), nucleotide-binding oligomerization domain-like (Nod-like) and C type lectin (CLRs) family receptors among others (Mogensen 2009).

1.2.2.1 Toll like receptor signalling

To date there are 11 TLRs identified in humans and 13 in mice, which are type 1 transmembrane proteins expressed primarily on sentinel cells (O'Neill, Golenbock et al. 2013). Some TLRs are found on the membrane, where they are capable of detecting PAMPs in the extracellular environment such as LPS, flagella, peptidoglycans of bacteria, lipoteichoic acid in gram-negative bacteria, lipoproteins and zymosan found in fungi (Hemmi and Akira 2005, Diebold 2009). Other TLRs are found intracellularly in the endosomal vesicles where they bind pathogenic nucleic acid such as single and double stranded RNA from viruses and CpG DNA of bacteria or fungi (Chaturvedi and Pierce 2009).

DC maturation is initiated via the uptake of antigens by TLRs, which elicits a cascade of intracellular signalling pathways, activating transcriptional factors such as activator protein 1 (AP-1), interferon-regulatory factors (IRF) and Nuclear factor-κB (NF-κB) (Rakoff-Nahoum and Medzhitov 2009, O'Neill, Golenbock et al. 2013). This leads to the production of cell signalling proteins and pro-inflammatory cytokines i.e., IL-12 and IFN- γ, increasing antigen presentation to naïve T cells, which in turn activates the antigen specific responses of the adaptive system (summarised in Figure 5) (Iwasaki and Medzhitov 2004, O'Neill, Golenbock et al. 2013).

1.2.2.2 NOD like receptor signalling

NOD like receptors (NLRs) are found as monomers in the cytosol of DCs where they act as intracellular sensors of PAMPs. These function similarly to TLRs and enhance TLR responses through the regulation of inflammatory and apoptotic responses. NOD 1 and 2 bind to a diverse range of peptidoglycan PAMPs and danger associated molecular patterns (DAMPS), such as proteins derived from the extracellular matrix (ECM) following tissue damage (Kawai and Akira 2009, Mogensen 2009). PAMPs detection via these NLRs activates NF-κB transcription factor leading to the production of cytokines, which are transported in endosomes and released into the extracellular space. This enhances the immune response generated (Franchi, Warner et al. 2009). However, NLRs are also capable of forming inflammasomes which activate Caspase-1, inducing the activation of cytokines pro-IL-1β and pro-IL-18, further amplifying the immune response (Shaw, Lamkanfi et al. 2010). Caspase-1 also leads to pyroptosis, a highly inflammatory programmed cell death, where there is an influx of extracellular fluid, cell swelling and membrane rupture. This in turn releases the inflammatory cytokines previously contained within the cell, which recruits further immune cells to the site of infection (summarised in Figure 5) (Jacobs and Damania 2012).

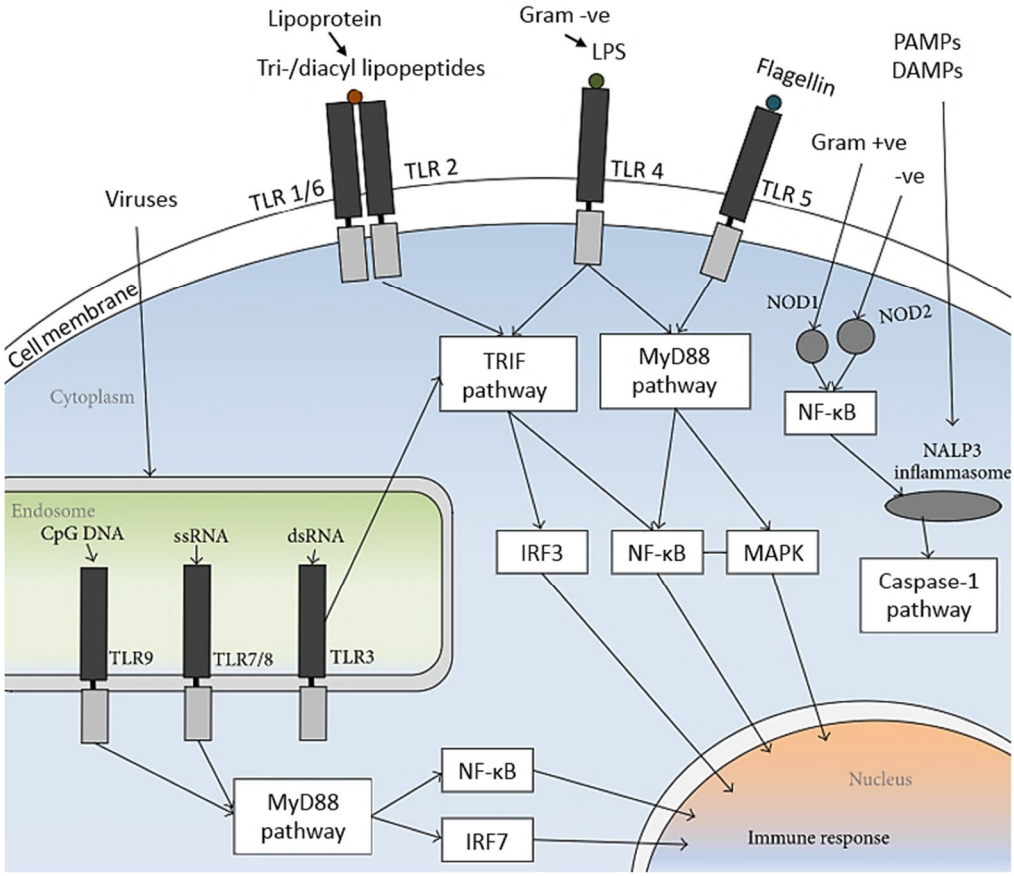


Figure 5: The schematic overview of NLRs and TLRs signalling pathways.

TLRs are involved in the recognition of PAMPs, from extracellular or endogenously processed pathogenic antigens. By binding to their respective ligands the receptor dimerizes and initiates intracellular signalling pathways. Through interactions with adapter molecules (i.e., Myeloid differentiation primary-response protein 88 and TIR domain-containing adaptor protein inducing IFNβ (MYD88 and TRIF)), downstream cellular interactions are initiated. Activating transcriptional factors such as activator protein 1 (AP-1), interferon-regulatory factors (IRF) and Nuclear factor-*κB* (NF-κB). In contrast NLRs act as intracellular sensors of PAMPs and DAMPs, which following antigen recognition, activates NF-κB transcription factors, functioning to enhance TLR responses. NLRs also activate the Caspase-1 pathway, leading to pyroptosis and further amplification of the immune response generated (Jang, Shin et al. 2015)

1.2.2.3 C type lectin receptor signalling

Due to their molecular structure, CLRs were mainly presented to differentiate Calcium $(Ca)^{2+}$ dependent, and Ca^{2+} independent carbohydrate binding lectins (Drickamer 1988, Zelensky and Gready 2005). CLRs expressed on DCs are specific for the recognition of glycosylated antigens. However signalling via CLRs alone does not necessary lead to the effective activation of T-cells, nevertheless it enables the APC function of DCs (Geijtenbeek and Gringhuis 2009). A conserved carbohydrate recognition domain (CRD) is found on all CLRs which determines the affinity of the CLRs to the carbohydrate residue. This receptor family has been categorized into two distinct groups based on their molecular structure (Drickamer and Taylor 2015). Group I transmembrane CLRs are part of the Mannose receptor (MR) family and multilectin protein group, including MR and DEC-205, which detect and bind mannosylated pathogenic units. Group II are the asiagloglycoprotein receptor family, including DC-SIGN, DC immunoreceptor (DCIR), C-type lectin-like receptor-1 (CLEC-1), DC-associated C-type lectin 1/2 (Dectin1 and 2), DC lectin (DLEC) and langerin on Langerhans cells (LC). Additionally there is also a soluble CLR, namely mannose binding lectin (MBL) (Figure 6) (Drickamer 1988, Figdor, van Kooyk et al. 2002).

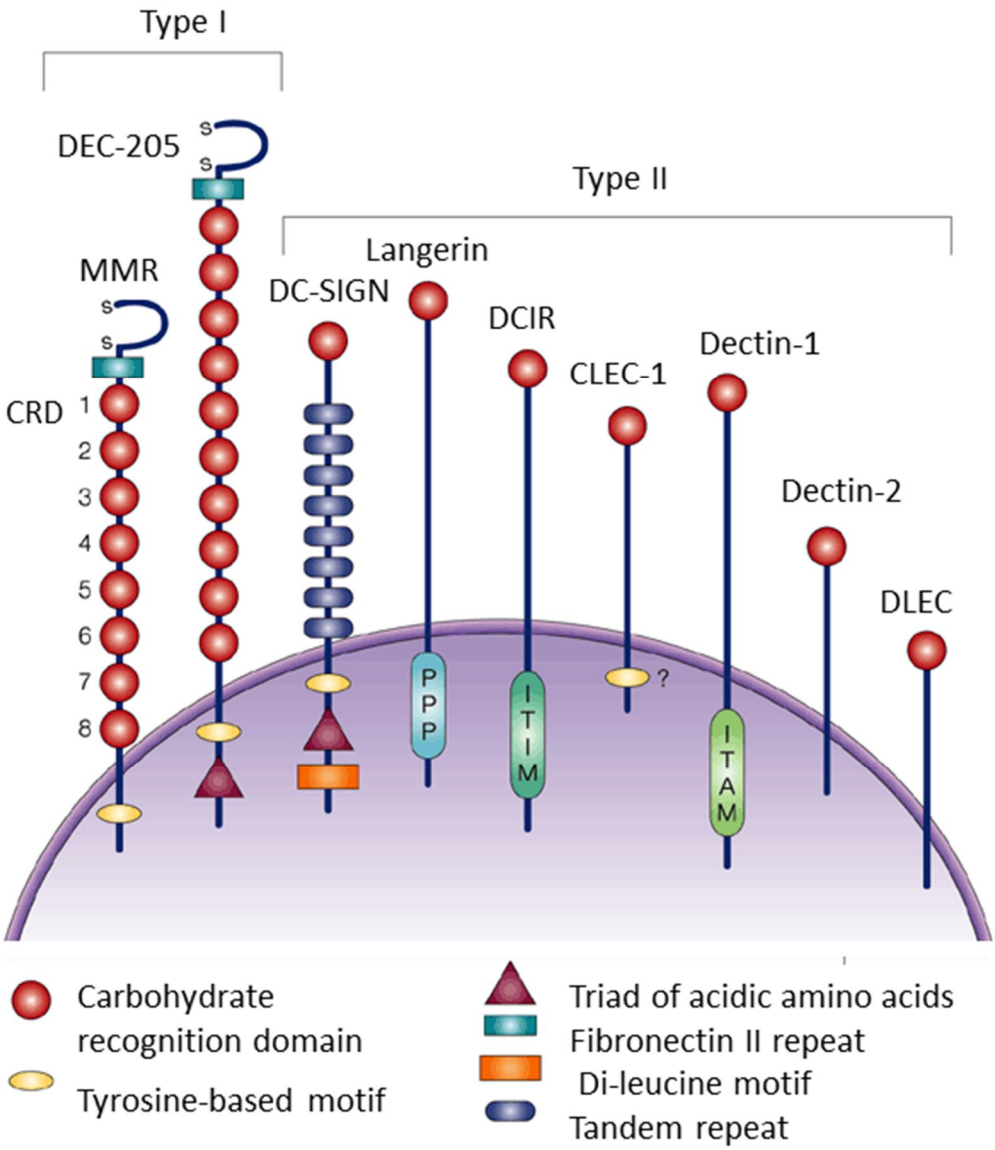


Figure 6: C type lectin receptors on dendritic and Langerhans cells.

Type I CLRs are comprised of an extracellular cysteine rich, amino terminal which has a single fibronectin type II domain, followed by 8 consecutive CRDs bound to an intracellular C-terminus. In contrast the type II CLRs are comprised of a single extracellular Carboxyl-terminal CRD, with a diverse cytoplasmic region between CLRs. These structures are conserved and necessary for antigen recognition (Figdor, van Kooyk et al. 2002).

Comparably, langerin present on LC in the epidermis also recognises glycosylated PAMPs in a Ca^{2+} dependent manner, sharing a substantial similarity in CRD binding regions with other CLRs such as MR and DC-SIGN (Feinberg, Powlesland et al. 2010). Albeit these similarities, langerin differs

through the capture, internalization, and effective degradation of antigens within Birbeck granules (Thepaut, Valladeau et al. 2009).

1.2.2.4 Pathogen recognition by MR

The MR is composed of an extracellular N-terminus, which has a single fibronectin type II domain, followed by 8 consecutive CRDs bound to an intracellular C-terminus (East and Isacke 2002, Figdor, van Kooyk et al. 2002). This receptor mediates the binding of pathogenic glycoproteins terminating in fucose, mannose or *N*-acetylglucosamine residues (Taylor, Gordon et al.). The recognition of these PAMPs elicits the complement system via the lectin binding pathway, enabling the endocytosis and phagocytosis of pathogenic microbes (Dodds 2002, Wallis 2007).

Primarily, the binding of MR to its respective PAMPs initiates the activation of MBL-associated serine proteases (MASPs). MASP-1 and 2 function to cleave complement proteins C4 and C2 into their subunits, namely C4b and C2a. These subunits, together with MASPs, form the C3 convertase (Schwaeble, Dahl et al. 2002, Wallis 2007). This process repeats as C3 convertase cleaves C3, with its C3b subunit binding the convertase effectively, and forming C5 convertase. The membrane attack complex is formed as the C5 complement protein is cleaved by its convertase, which produces and binds C5b, recruiting C6, C7, C8 and C9.

These components assemble to generate a pore in the cell membrane, leading to the rupture and lyses of the pathogen (Kerr 1980, Wallis 2007).

The MR also functions as a PRR during endocytosis where the receptor binds exogenous mannosylated pathogens, and the MR-ligand complex is internalised through clathrin coated pits. These are then transported to endocytic compartments, where the MR releases the ligand in the acidic endosome environment. This enables the MR to be recycled consecutively to the cell surface and mediates further ligand internalization (Stahl, Schlesinger et al. 1980). The antigens are digested into peptide fragments and eventually co-expressed with Major histocompatibility complex (MHC) class II on the cell surface, where it is presented to $CD4^+$ T cells (Sallusto, Cella et al. 1995, Engering, Cella et al. 1997).

Nonetheless, MR phagocytic capabilities have been questioned, with some cell lines unable to internalise known ligands (Le Cabec, Emorine et al. 2005). In contrast, other studies have reported the presence of MR on phagosomes, encapsulating *C. albicans* in human DCs (Cambi, Netea et al. 2008). These are some contradictory findings in respect to MR phagocytic capabilities. It is therefore presumed that MR is a non-professional phagocytic receptor, requiring a receptor-complex for signal transduction and effective pathogen uptake (Le Cabec, Emorine et al. 2005). However, due to the lack of detailed knowledge on the cytoplasmic signalling of MR phagocytosis, the exact phagocytic signalling mechanism remains to be elucidated in more detail.

Intriguingly, studies in other helminth infections (*Schistosoma mansoni*) revealed a novel pathway involving the internalization of helminth derived glycoproteins through ligation of the MR. This interaction has been shown to interfere with DC protein synthesis, conditioning the immune cells for a Th2 phenotype (Paveley, Aynsley et al. 2011, Everts, Hussaarts et al. 2012). In the context of Necatoriasis, albeit the surface of the *N. americanus* larvae sheath is heavily glycosylated (Kumar and Pritchard 1992) the potential role for this surface-chemistry in subverting DC function through MR-ligand interactions remain to be investigated.

1.2.2.5 Pathogen recognition by DC-SIGN

DC-SIGN is a multi-functional CLR involved in pathogen recognition (van Kooyk and Geijtenbeek 2003), intracellular signalling, cell adhesion/migration (Geijtenbeek, Krooshoop et al. 2000) and antigen presentation (van Vliet, den Dunnen et al. 2007). This CLR is primarily expressed by immature DCs in the peripheral tissues, and is also found on inflammatory macrophages in the dermis and intestinal mucosa. However DC-SIGN is significantly downregulated on mature DCs in the lymphoid tissues (Geijtenbeek, Krooshoop et al. 2000, Granelli-Piperno, Pritsker et al. 2005). Structurally DC-SIGN is comprised of four regions, namely a single Ca^{2+} dependent extracellular C-terminal CRD, connected to a neck repeat-region composed of hydrophobic amino acid residues

(Geijtenbeek, Krooshoop et al. 2000). This is followed by the transmembrane domain and ended by an intracellular N-terminal cytoplasmic tail (Figure 6) (Feinberg, Mitchell et al. 2001, Mitchell, Fadden et al. 2001, Figdor, van Kooyk et al. 2002).

As a PRR, DC-SIGN has a capacity for recognizing and binding mannose, fucosylated glycans, and *N*-acetylglucosamine on a range of pathogens due to its EPN (Glu-Pro-Asn) motif (Mitchell, Fadden et al. 2001, Appelmelk, van Die et al. 2003). However, depending on the carbohydrate group expressed by the pathogen, distinct signalling pathways are initiated via DC-SIGN binding (Gringhuis, den Dunnen et al. 2009). Prior to the recognition of pathogenic glycans, the intracellular tail of DC-SIGN is bound to the adaptor protein, lymphocyte specific protein 1 (LSP-1), associated with a triad RAF-1 signalosome. The signalosome consists of, connector enhancer of kinase suppressor Ras1 (CNKSR1), kinase suppressor of Ras (KSR) and RAF proto-oncogene serine/threonine-protein kinase (RAF) (Gringhuis, den Dunnen et al. 2009, Williams 2009).

Recognition of pathogens encompassing mannose residues, such as mycobacteria and HIV-1, triggers the signalosome to acts as a docking site for recruiting upstream effector factors, which in turn phosphorylates RAF-1. Activation of the pathway downstream of RAF-1 results in the acetylation and phosphorylation of the p65 subunit, which together with p50 forms the NF-κB heterodimer complex (Gringhuis, den Dunnen et al. 2007). This pathway is

fundamental for DC-SIGN immune response modulation of TLR-4 induced NF-κB activation, through the regulation of the p65 subunit. The activated p65 subunit has prolonged activity and an enhanced stability. This in turn increases the rate of transcriptional factors promoting proinflammatory cytokines, IL-12a, IL12b, IL-6 and Th_1/Th_{17} polarization (Figure 7) (Gringhuis, den Dunnen et al. 2007, Gringhuis, den Dunnen et al. 2009, Geijtenbeek and Gringhuis 2016).

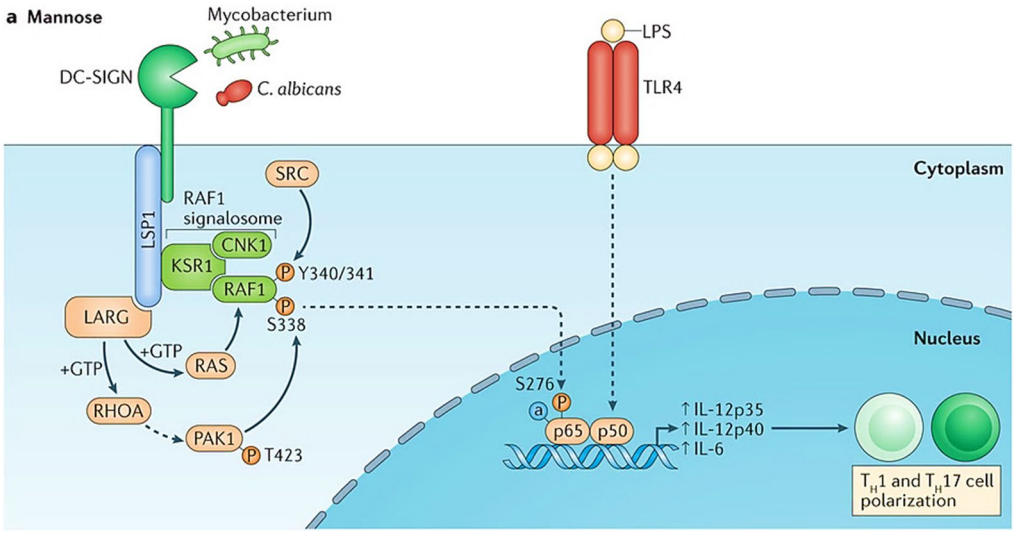


Figure 7: Mannose-carbohydrate specific immunity by DC-SIGN signalling.

Recognition of pathogens with mannose residues triggers the triad-signalosome to acts as a docking site for recruiting upstream effector factors. This phosphorylates RAF-1 and activates downstream pathways resulting in the acetylation and phosphorylation of the p65 subunit. Together with p50, the NF-κB heterodimer complex is formed. The activated p65 subunit has prolonged activity and an enhanced stability, increasing rate of transcriptional factors, proinflammatory cytokines IL-12a, IL12b, IL-6 and promotes Th_1/Th_{17} polarization (Geijtenbeek and Gringhuis 2016).

In contrast, the detection of fucose in PAMPs instigates RAF-1 signalosome to actively dissociate from LSP-1 adaptor protein, which remains bound to DC-SIGN. This triggers the phosphorylation of LSP-1 through TLR4-PRR mediated

activation of mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 (MK2) (Gringhuis, den Dunnen et al. 2009). This pathway leads to the modulation of TLR4 induced immune responses through the regulation of B cell lymphoma 3 (BCL-3), via the specific fucose signalosome, which can positively/negatively regulate NF-κB activation (Gringhuis, Kaptein et al. 2014). The de-ubiquitinase CYLD component of the signalosome promotes the translocation of ubiquitylated BCL-3 to the nucleus. Additionally, this leads to the expression of IFN-β, which activates the signal transducer and activator of transcription 1 (STAT-1) pathway. In contrast, the IκB kinase subunit-ε (IKKε) prevents this accumulation. Furthermore, the translocation of BCL-3 promotes a Th2 polarization and enhances chemokine expression of CCL17, 22 and anti-inflammatory IL-10 with a suppression in pro-inflammatory cytokines produced in response to mannose recognition of DC-SIGN (Figure 8) (Gringhuis, Kaptein et al. 2014, Geijtenbeek and Gringhuis 2016).

Interestingly, a shared feature among many parasitic helminths includes the ability to inhibit Th1 responses, and polarise the immune response towards a Th2 differentiation (Loukas and Prociv 2001, Geiger, Caldas et al. 2007, McSorley and Maizels 2012, McSorley, Hewitson et al. 2013). Several studies have speculated that nematodes and helminths are exploiting this alternative route through DC-SIGN-ligand interactions (Gringhuis, den Dunnen et al. 2009, Klaver, Kuijk et al. 2015). However in regards to *N. americanus* larvae, this remains elusive with no knowledge on whether the glycosylated sheath interacts with these DC-SIGN or MR.

Furthermore, the activation of IFN-β induced Janus kinase 1 (JAK1) expression, phosphorylates STAT1 which couples with STAT2 and IFN-regulatory factor 9 (IRF9) in the nucleus. This promotes the transcription of IL-27, effectively mediating T follicular helper cell (T_{FH} cell) differentiation. Overall, the ability to distinguish the immune response generated based on the pathogenic antigens encountered (mannose or fucose), is a unique function of DC-SIGN within the CLR family (Gringhuis, den Dunnen et al. 2009, Gringhuis, Kaptein et al. 2014).

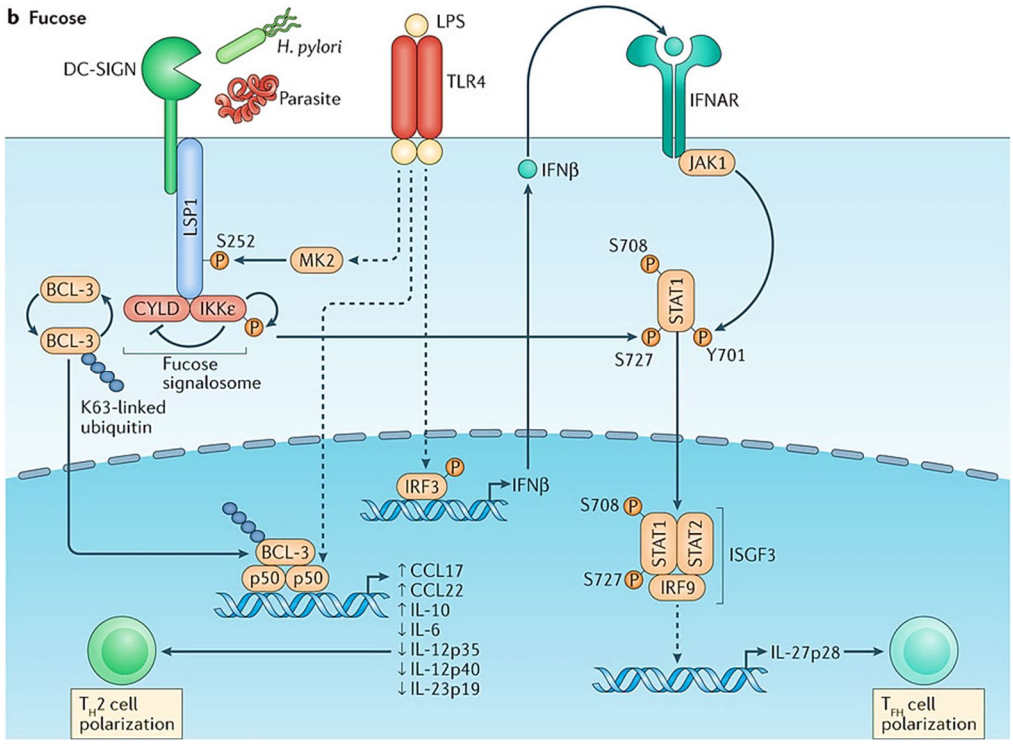


Figure 8: Fucose-carbohydrate specific immunity by DC-SIGN signalling.

Recognition of pathogens with fucose residues triggers the triad-signalosome to dissociate from the DC-SIGN bound LSP-1 adaptor protein, which becomes phosphorylated, activating MK2 through TLR4. This leads to NF-κB activation which is regulated by components of the signalosome, through the promotion or inhibition of BCL-3 localization in the nucleus, by CYLD and IKKε respectively. The accumulation of BCL-3 in the nucleus promotes Th2 polarization, chemokine expression of CCL17, 22 and anti-inflammatory IL-10

with a suppression in pro-inflammatory cytokines produced in response to mannose recognition of DC-SIGN (Geijtenbeek and Gringhuis 2016).

Finally, both MR and DC-SIGN have been shown to functions as adhesion molecules. The latter has a high affinity for intracellular adhesion molecule (ICAM)-3 to support DC interactions with T cells, and ICAM-2 which promotes trans-endothelial migration (Geijtenbeek, Krooshoop et al. 2000). Furthermore, *Borrelia burgdorferi* has been shown to employ the MR to adhere firmly to monocyte derived macrophages and MR-transfected rat cells (Cinco, Cini et al. 2001). Additionally, this CLR on fibroblasts has also been shown to bind collagen through its fibronectin domain, identifying the participation of MR in adhesion (Napper, Drickamer et al. 2006).

1.2.2.6 T Cell priming by mature dendritic cells

Following exposure to *N. americanus* larvae, no protective immunity develops in the host. This has been linked to an inability of DCs to efficiently stimulate naïve T cells, which then hampers the activation of appropriate adaptive responses (Loukas and Prociv 2001, Fujiwara, Cancado et al. 2009). Following antigen encounter, DCs express peptide fragments generated from the invading pathogen in conjunction with the major histocompatibility class (MHC) I or II (Blum, Wearsch et al. 2013).

1.2.2.6.1 MHC I

In the case of infected cells, the pathogen synthesises endogenous proteins, which are degraded into smaller fragments through the cytosolic proteasome. These small fragments then enter the endoplasmic reticulum (ER) through the TAP1 and 2 transporters, where they are further broken down (Antoniou, Ford et al. 2002, Blum, Wearsch et al. 2013). The ribosomes in the ER synthesise an incomplete MHC I molecule consisting of the alpha 1, 2 and 3 domains. This structure is then bound to the chaperone protein calnexin, following which macroglobulin beta 2 binds, effectively completing the MHC I molecule (Chaplin 2010). Calnexin chaperones the MHC I to the TAP transporters, but in combination with erp57, covers the variable alpha region of the MHC I molecule. This prevents peptide fragments from binding to this groove (Diedrich, Bangia et al. 2001, Wearsch and Cresswell 2007). Here the construct interacts with tapesin, causing erp57 and calnexin to dissociate from the complex, allowing antigenic peptides to bind. The MHC I then leaves the ER via an endosome through the Golgi apparatus, and localises to the cell surface where it is presented to $CD8^+$ T cells (Hulpke, Baldauf et al. 2012, Blum, Wearsch et al. 2013).

The T cell receptor (TCR) on $CD8^+$ T cells recognises and binds to the MHC I/antigen complex. Subsequently, the CD8 receptor binds to the α3 domain of the MHC I molecule, to further strengthen interaction and ensure activation (Daniels, Devine et al. 2001, Belyakov, Kozlowski et al. 2007, Pennock, White

et al. 2013). This activates the secondary signal, where CD28 on the T cell binds the co-receptors CD80 and CD86 on the APC. Once the $CD8^+$ T cells recognise the antigen in the presence of IFN-α/β and IL-12, the cell is rendered cytotoxic (Tc) (Pennock, White et al. 2013). Perforin is released by the Tc, which creates pores in the membrane of the infected cells, through which serine proteases enter. The serine protease, granzyme-A, cleaves NADPH in the mitochondria to produce toxic reactive oxygen species (ROS), and granzyme-B activates the caspase cascade, initiating cell apoptosis through lysis (Trapani and Smyth 2002, Pennock, White et al. 2013). Furthermore, the activated Tc Fas ligand (CD95L) can also bind to its Fas (CD59) receptor, on the infected APC. A death inducing signalling complex (DISC) is formed through the autocatalytic cleavage activity of procaspase-8, and Fas (CD95) associated protein with death domain (FADD) (Kischkel, Hellbardt et al. 1995, Strasser, Jost et al. 2009, Paulsen and Janssen 2011). Cytokines produced by Tc include IL-2, promoting further activation and proliferation of $CD8^+$ T cells, and IFN- γ/TNF-α, which can also stimulate naïve $CD4^+$ cells into their Th1 subset (Pennock, White et al. 2013).

1.2.2.6.2 MHC II

In contrast, MHC II is $CD4^+$ T cell restricted and processes exogenous antigens following phagocytosis. These are engulfed and degraded in the acidic environment of the phagolysosome (Gao, Rao et al. 2002, Savina and Amigorena 2007, Flannagan, Cosio et al. 2009). This then stimulates the ribosomes within the ER to synthesis MHC II, consisting of 2 alpha and beta domains, which

initiates a cascade of events (Jones, Fugger et al. 2006). Primarily, the chaperone Ii protein (CD74) interact with the MHC II, preventing premature peptide loading. This chaperone also guides the MHC II in an endosome, moving through the ER and Golgi apparatus (ten Broeke, Wubbolts et al. 2013). The endosome has an acidic environment which degrades Ii, leaving a smaller protein fragment (CLIP) to cover the binding site. (Watts 2012, ten Broeke, Wubbolts et al. 2013). The protein HLA-DM interacts with MHC II and CLIP is released, allowing the pathogenic peptides to bind (Schulze and Wucherpfennig 2012). This complex is then expressed on the cell surface, where it is presented to $CD4^+$ T cells (Blum, Wearsch et al. 2013).

During this process, TCR-CD3 binds to the α1 β1 domain of the MHC II/peptide complex. CD4, a co-receptor, also binds to the MHC II, but at the β_2 region. This initiates the phosphorylation of the CD3 cytoplasmic conserved domain, through the Src family tyrosine kinase (Lck), bound to the cytoplasmic tail of CD4. Once phosphorylated, the SH2 domain containing protein kinase ZAP-70 is recruited to the immunoreceptor tyrosine-based activation motif (ITAM) (Lineberry and Fathman 2006, van der Merwe and Dushek 2011). The secondary signal also employs CD28 on $CD4^+$ T cells, binding to co-receptors CD80 and CD86 on APC. The naïve $CD4^+$ T cells mature into different effector cell sub types, which are classed under Th1, Th2, Treg or Th17 cells (Zhu, Yamane et al. 2010). These interactions are necessary to activate intracellular transcriptional factors such as NF-κB, consequently initiating T cell responses. The activation of T cells by

DCs is summarised in Figure 9 (Wang, Matsumoto et al. 2004, Thaker, Schneider et al. 2015).

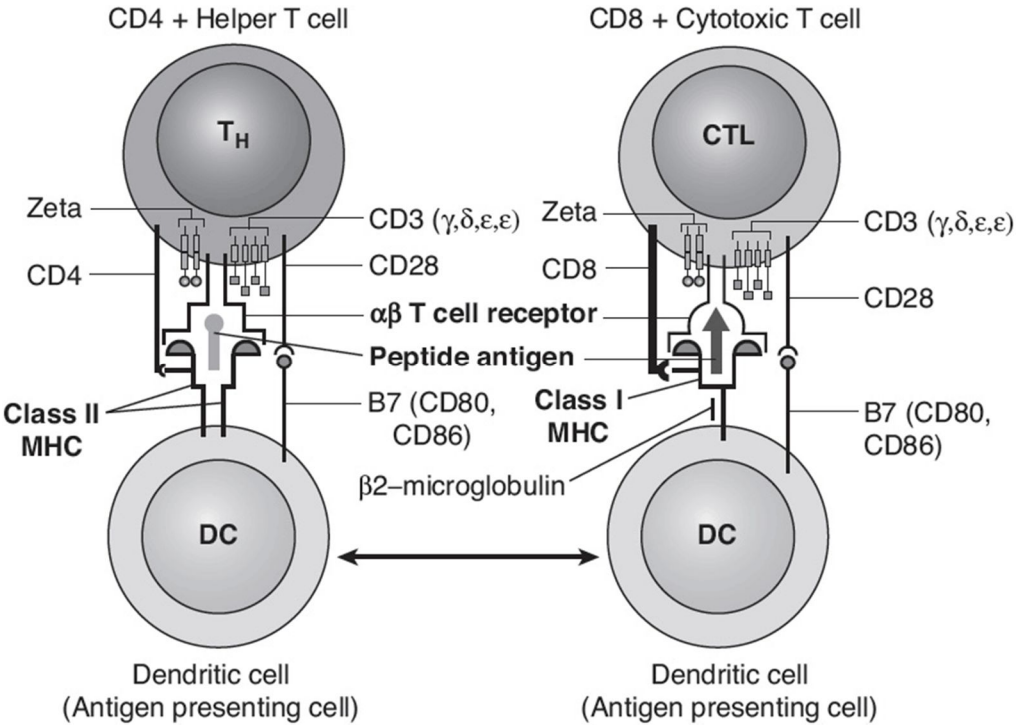


Figure 9: T cell activation by DC MHC-peptide complex presentation.

Naïve $CD4^+$ T cells process exogenous antigens following phagocytosis, which are presented in complex with MHC II on the surface of DCs. The TCR-CD3 binds to α1 β1 domain of the MHC II/peptide complex and CD4 binds at the β_2 region. The secondary signal, CD28, binds to co-receptors CD80 and CD86 on the DCs, following which the naïve $CD4^+$ T cells mature into different effector cell sub types. In contrast, naïve $CD8^+$ T cells are primed by endogenous peptides which are processed and presented in complex with MHC I on the surface of DCs. TCR on $CD8^+$ T cells binds to MHC I/antigen complex, following which the CD8 receptor binds to the α3 domain of the MHC I molecule. The secondary signal, CD28 binds to co-receptors CD80 and CD86 on the DCs and in the presence of IFN-α/β and IL-12, the cell is rendered cytotoxic (Blum, Wearsch et al. 2013).

From: https://basicmedicalkey.com/immunology-4/

Th1 polarization is generated in response to intracellular bacteria and protozoa, where the cells differentiate and secrete a range of cytokines including IFN-γ, IL-2 and TNF-β. IL-2 acts directly on Th cells in a self-promoting manner, through the IL-2 receptor and IFN- γ promotes further Th1 polarization (Kaiko, Horvat et al. 2008). The latter also serves as a primary signal for the activation of macrophage phagocytosis, followed by the secondary signal, binding T cell CD40L to the macrophage CD40 (Elgueta, Benson et al. 2009).

In contrast, Th2 responses are directed at expelling extracellular parasites including *N. americanus*, characterized by an expression in cytokines IL-4, 5, 10 and 13 (Kaiko, Horvat et al. 2008, Zhu, Yamane et al. 2010). Comparably, IL-4 is a positive feedback for Th2 cells, where this cytokine directly acts in a self-promoting manner, in addition to stimulating mast cells through the activation of B-cell mediated IgE production (Zhu, Yamane et al. 2010). The de-granulation of mast cells results in the release of histamine, leukotriene and serotonin. Collectively, these inflammatory mediators act to induce the acidification of the gastric fluid and stimulate intestinal peristalsis.

Continuing, an overexpression of IL-5 stimulates B cell differentiation and the production of antibody secreting plasma cells (Kaiko, Horvat et al. 2008). However it is also a principal maturation factor for eosinophils and basophils, increasing the level of inflammatory cells recruited to the site of infection (Kouro and Takatsu 2009). Considering the consequences in Th2 mediated cell immunity, an over activation results in a variety of autoimmune diseases and

hypersensitivity. Necatoriasis in particular is associated with type 1 hypersensitivity, which is characterised by the production of IgE antibodies, instead of IgA, where it binds to the Fcε on mast cells during sensitisation (Galli and Tsai 2012). A secondary or later exposure to the same antigen will then result in IgE bound to the mast cells, to cross link with the allergen, effectively activating and stimulating the secretion of inflammatory mediators (Larche, Akdis et al. 2006, Galli and Tsai 2012). Following clonal expansion and proliferation, both B/T cells differentiate into a subset of memory cells with a prolonged life-span; maintaining the antigen affinity of the initial activation signal (Farber, Yudanin et al. 2014, Kurosaki, Kometani et al. 2015).

As detailed previously, the prevalence and rate of reinfection associated with human Necatoriasis, highlights the need for further research focusing on the initial stages of infection following exposure (Loukas and Prociv 2001). This thesis has examined the initial interactions between cells of the innate anatomical barrier i.e., keratinocytes, and L_3 *N. americanus* larvae. In addition, a 3D immunocompetent model of human skin was developed. Therefore the following will introduce the skin and its functions in maintaining a protective epithelial barrier.

1.3 The skin overview

The human skin is of great importance as it provides a direct interface between the body and the physical environment. This anatomical layer acts as a multifunctional defence barrier against physical, chemical and microbial factors (Simpson, Patel et al. 2011, Baroni, Buommino et al. 2012). Nevertheless, this barrier is rendered ineffective in the prevention of hookworm infection, as the *N. americanus* larvae are capable of breaching the epidermal and dermal layers (Matthews 1982, Loukas and Prociv 2001). The skin when intact encompasses an effective permeability barrier, permitting the selective passage of molecules that are ≤40 nm (Brandner, Zorn-Kruppa et al. 2015). This then highlights the apparent size of L_3 *N. americanus* (300-400μm) and how it could possibly gain access to the host vasculature.

The average skin of an adult ranges from 1.5-2 m^2 in surface area, and 2-3 mm in thickness, making it the largest organ of the human body (Bouwstra, Honeywell-Nguyen et al. 2003, Simpson, Patel et al. 2011). It regulates body temperatures (i.e., insulation, thermoregulation) via the surface blood flow, and prevents dehydration caused by excessive water loss (Baroni, Buommino et al. 2012, Boer, Duchnik et al. 2016). The skin is a key component enabling the response to external stimuli, such as pressure, and has absorptive functions in reducing damage caused by ultraviolet (UV) radiation (D'Orazio, Jarrett et al. 2013, Lloyd, McGlone et al. 2015). Overall it consist of 2 major sections; the epidermis, which acts as the anatomical barrier resting on a layer of ECM, and

the dermis underneath providing nutrient diffusion and structure (Di Meglio, Perera et al. 2011). This layer of specialised ECM functions as a physical boundary and dynamic interface, between the two skin compartments, establishing the basement membrane (basal lamina) (Breitkreutz, Koxholt et al. 2013).

1.3.1 Epidermis

The epidermis is the outermost layer of the skin which encompasses an adhesive network of keratinocytes in different developmental stages, making it a stratified squamous epithelium (Baroni, Buommino et al. 2012). Notably, there are no blood vessels in the epidermis, as cells in the deeper layers of the skin are nourished via blood capillaries present in the dermoepidermal junction (Fuchs and Raghavan 2002). The epidermis comprises a cyto-architectural assembly, which can be segregated into 4 distinct layers of strata. These are the stratum corneum followed by the stratum granulosum, stratum spinosum and finally the lowest layer of the epidermis, the stratum basale (Figure 10) (Baroni, Buommino et al. 2012).

The cells in the basal lamina are arranged in a vertical profile where they attach to the underlying basement membrane via hemidesmosomes. These are asymmetrical in structure and bind to the epithelial inner basal surface plaque, through integrins and adhesion molecules, functioning to anchor the cells (Simpson, Patel et al. 2011, Breitkreutz, Koxholt et al. 2013). The cells in this

layer are in a state of continues asymmetric mitosis, proliferating and migrating superficially towards the stratum spinosum (Bouwstra, Honeywell-Nguyen et al. 2003). As the keratinocytes continue to become stratified, the decreased integrin density, permits cell cycle exit, following which the cells begin to enlarge and develop intracellular connections (Simpson, Patel et al. 2011). Once the cells reach the stratum granulosum, they become flattened and form cornified membranes, commencing the loss of both nuclei and cytoplasmic organelles, prior to differentiation into keratinized squames. (Bouwstra, Honeywell-Nguyen et al. 2003, Simpson, Patel et al. 2011). Intracellular organelles are degraded through the secretion of lysosomal enzymes by keratinocytes. The cells in the corneal layer are tightly crosslinked and densely packed together, forming a squamous layer, collectively establishing a functional external barrier (Simpson, Patel et al. 2011).

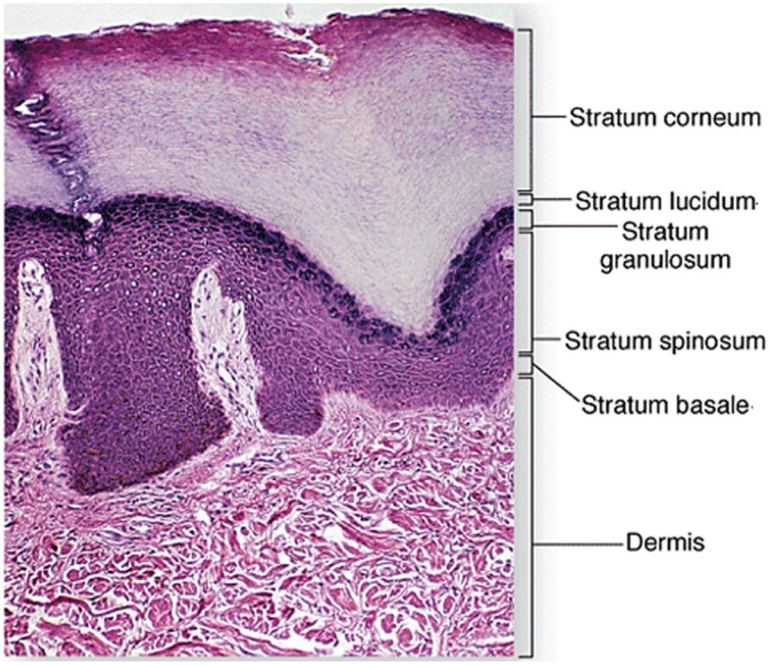


Figure 10: Layers (strata) of epidermis and dermis in thick skin.

The skin consist of 2 main structures; primarily the epidermis which constitutes the stratum corneum, lucidum, granulosum, spinosum and basale. The epidermis acts as the anatomical barrier, which rests on a layer of ECM in the stratum basale, separating it from the dermis underneath.

Adapted from Junqueira's basic histology, 13th edition, Chapter 18: http://accessmedicine.mhmedical.com/content.aspx?bookid=574&sectionid=42524604

Furthermore, although 80% of the epidermis cell population are predominantly keratinocytes, there are other resident cells present within this construct (Di Meglio, Perera et al. 2011). For instance, melanocytes are localised within the basement membrane, functioning to absorb the damage caused by UV radiation (D'Orazio, Jarrett et al. 2013). The skin melanocytes synthesise melanosomes, and produce melanin pigment inside these vesicles. These attach to the continuously dividing keratinocytes, and provide the skin with pigmentation (Wasmeier, Hume et al. 2008, Cichorek, Wachulska et al. 2013).

Other essential cells present in the skin are the LC, which function as immature DCs in the epidermis and mucosal tissues (Valladeau, Ravel et al. 2000, Mc Dermott, Ziylan et al. 2002). These immune cells can be found throughout all the layers in the epidermis, however they are most commonly localized to the stratum spinosum (Jaitley and Saraswathi 2012). The LC have a morphological and functional similarity to myeloid DCs, and express the mannose, fucose and *N*-acetylglucosamine binding CLR, langerin (Condaminet, Peguet-Navarro et al. 1998, Valladeau, Ravel et al. 2000). LC also function to take up and process foreign pathogenic antigens in the peripheral tissues, followed by the migration to the regional lymph nodes, where they prime naïve T cells (Kripke, Munn et al. 1990, Moll, Fuchs et al. 1993).

1.3.2 Dermal-epidermal junction

The dermoepidermal junction is the acellular tissue that joins the epidermal and dermal layers of the skin, via anchoring filaments of hemidesmosomes. This junction, also referred to as the cutaneous basement membrane zone, is comprised of 4 layers. These are the sub-basal membrane plaque, lamina lucida, lamina densa and the fibrillary zone (Briggaman and Wheeler 1975, Breitkreutz, Koxholt et al. 2013). Collagen IV and laminin are the main mechanical scaffolds present in this layer (Nguyen, Gobinet et al. 2012), which interlock together via collagen composed fibrils and anchoring plaques, to the dermis (Breitkreutz, Koxholt et al. 2013). This structure provides the epidermis with functional and mechanical support, preserving the integrity of the dermal-epidermal junction (Wickett and Visscher 2006).

1.3.3 Dermis

The dermis is the densest layer in the skin, which encompasses a complex vascular network of connective tissues derived from dermal fibroblasts (Baroni, Buommino et al. 2012). This network provides blood and lymphatic vessels for the elimination of waste and transport of nutrients. However, it also contains sweat, sebaceous and apocrine glands in addition to hair follicles and mechanoreceptors (Baroni, Buommino et al. 2012). Structurally, this layer is divided into two segment; the superficial papillary region and the deep reticular

region (Wickett and Visscher 2006). The dermis is predominantly comprised of collagen types I and III, as these have a resistance to proteolytic degradation and function to maintain the skin structure and stability. Other fibril collagens in the dermis, i.e., types V, VI and VII, function as fibril bridges anchoring the polypeptide fibres to components of the ECM (Wickett and Visscher 2006, Breitkreutz, Koxholt et al. 2013). The subcutaneous vascular network in the dermis has diverse functions as it regulates the temperature in the skin. This plexus responds to sympathetic stimuli, regulating the vasoconstriction and dilation of cutaneous blood vessels (Wickett and Visscher 2006). In addition, the lymphatic vessels in the dermis contain dendrocytes, mast cells, macrophages and LC, functioning to protect the host against a range of microbial pathogens (Skobe and Detmar 2000, Grice and Segre 2011).

1.3.4 Extracellular matrix

Fibroblasts constitute the majority of cells in the dermis, which are generally localized in the papillary region. These cells synthesise the connective tissue fibres found within the ECM, and are actively engaged in essential paracrine signalling interactions, with other resident cell types (i.e., epithelial cells) (Cantòn, Cole et al. 2010, Wojtowicz, Oliveira et al. 2014, Tracy, Minasian et al. 2016). The growth factors secreted by the dermal fibroblasts are fundamental for the stratification and normal morphogenesis of the epidermis, highlighting the importance of stromal-epithelial interdependency (Ornitz and Itoh 2015, Sriram, Bigliardi et al. 2015). Furthermore, literature has shown their essential role for depositing a range of structural proteins and collagen into the ECM

during wound healing. (Tracy, Minasian et al. 2016). Overall, dermal fibroblasts are crucial components, necessary for the cellular adhesion, growth, stability and organization of the ECM (Tracy, Minasian et al. 2016).

The ECM provides the skin with its structural integrity, comprised of connective tissues, structural fibres and an interlocking ground substance (Simpson, Patel et al. 2011, Breitkreutz, Koxholt et al. 2013). The ground substance is made of water and glycosaminoglycans that are bound to core proteins in the form of proteoglycans. It is a structure that permits the diffusion of nutrients between neighbouring tissues (Hynes 2009), whilst providing gel-like adhesion bridges between neighbouring cells. This aids the regulation of healing, inflammation and cancerous developments (Lam, Truong et al. 2014).

In this context, *N. americanus* larvae have been reported to secrete proteolytic enzymes capable of degrading components of the ECM, including elastin, collagen, fibronectin and laminin (Brown, Girod et al. 1999). Previous studies have reported various nematodes to disrupt the tight junction barrier of epithelial cells during disease. For instance, *in vivo* mouse models of *Trichinella spiralis* infection, reported critical mastocytosis, which resulted in disrupted intestinal barrier integrity (McDermott, Bartram et al. 2003). In addition, recent studies on *N. americanus* larval proteases, reported the disruption of vascular endothelial cadherin and F-actin (Souadkia, Brown et al. 2010). However, no such studies have been investigated in key tight junction proteins of cells which primarily encounter the parasitic hookworms, namely epidermal keratinocytes. Therefore the following will introduce the role of epithelial tight junctions (TJs) in the skin.

1.3.5 The epithelial barrier

The multifunctional epithelial barrier functions to preserve the transfer of ions, water and macromolecules through the paracellular pathway. The intracellular pathway, facilitates the passage of lipids and proteins through the apical and basolateral domains (Grice and Segre 2011, Brandner, Zorn-Kruppa et al. 2015). This construction is paramount in establishing the skin's structural integrity and epithelial permeability, necessary for the compartmentalization between tissue spaces across the epithelium. It is a structure comprised of a range of transmembrane proteins, which attach to the cytoskeleton creating a complex cell-cell interaction network between surfaces and the sub-stratum (Tokuda, Miyazaki et al. 2009).

The transcellular pathway is regulated through primary and secondary active transport, enabling the movement of molecules via electrochemical potential differences (i.e., charged ions and macromolecules) permitting diffusion against the concentration gradient (Anderson and Van Itallie 2009). Some molecules are transported via transcytosis, where the particle is encapsulated in a transport vesicle across the cell (Tuma and Hubbard 2003). Finally, there are ion channels/pumps dispersed asymmetrically across the apical and basolateral membranes, providing further transcellular movement (Günzel and Yu 2013).

In contrast, the movement of molecules in the paracellular pathway is regulated by essential TJ proteins and subjacent adherens junctions (AJ). These bridge the

apical intercellular space between the epithelial cells, forming the apical junctional complex (JC) (Anderson and Van Itallie 2009). This barrier has been associated with the regulation of various transcription, proliferation and polarity activities within the cell, contributing towards epithelial homeostatic balance, and physiological functions (Cereijido, Valdes et al. 1998, Förster 2008). Paracellular permeability and the assessment of its integrity within the epithelium is therefore a fundamental measure. Barrier function is principally evaluated through biophysical properties via the measurement of transepithelial electrical resistance (TEER) applying the Ohm's law. This measures the difference in voltage between the basal and apical compartments of the cell layer. The voltage is characterized by the ionic current in the media, continuously passing through both pathways across the epithelium (Srinivasan, Kolli et al. 2015).

There are three main different types of cell connecting junctions found within the epithelium, which constitute the TJ, AJ, desmosomes and gap junctions (GJ). The latter provides essential intracellular passageway for molecules between cells (Anderson and Van Itallie 2009). In this thesis human epidermal keratinocytes were differentiated to develop a functional barrier, *in-vivo*, confirmed by the expression of tight junction protein Zonula occludens (ZO-1).

Primarily, the in depth functions of the components which are involved in mammalian skin barrier formation require further clarification, specifically in relation to studies with diseased epidermis (Brandner, Zorn-Kruppa et al. 2015).

Nevertheless, both occludin and claudin are considered integral TJ proteins, with claudins as the major constituents of the TJ strands (Chiba, Osanai et al. 2008). These bind to peripheral membrane proteins, connecting the actin in the cytoskeleton of neighbouring epithelial cells, establishing the paracellular pathway integrity. Following which the actin cytoskeleton associates with, and binds TJ proteins such as ZO, including ZO-1, -2, -3, in the cytosol (Itoh, Furuse et al. 1999, Chiba, Osanai et al. 2008, Anderson and Van Itallie 2009). The basic structure of key tight junction components in epithelial cells is illustrated in Figure 11.

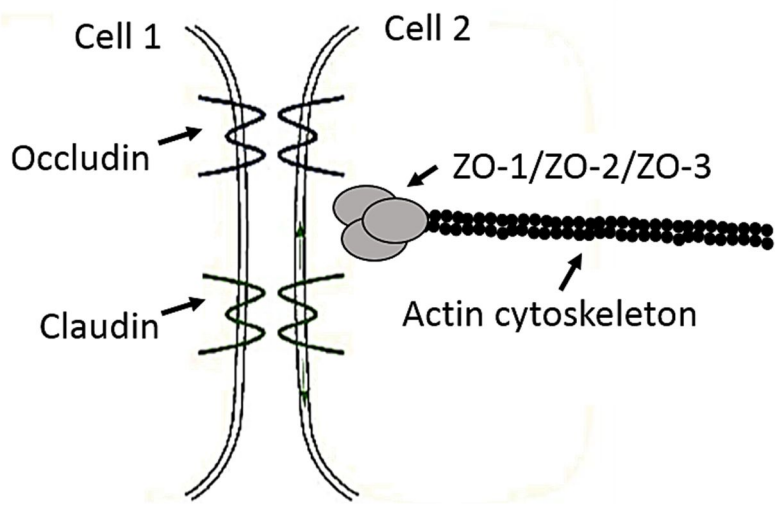


Figure 11: The molecular structure of key epithelial cell to cell junctions.

The ZO proteins crosslink occludin and claudin with cytosolic proteins, effectively bridging the TJ strands formed to the cytoskeleton. Adapted from: (Steed, Balda et al. 2010)

1.4 Thesis Plan

Chapter 1 introduced the research presented in this thesis and the scientific motivation behind the work conducted. The overall aim of the project is to elucidate the early events between L_3 *N. americanus* infective larvae and components of the innate immune system particularly, DCs and epidermal keratinocytes. In order to gain a better understanding of the early events surrounding the initial stages of infection in Necatoriasis and identify new mechanisms by which *N. americanus* regulates immune responses, the following objectives were investigated.

i. The physical interaction between L_3 *N. americanus* larvae and human DCs and epidermal keratinocytes.

ii. The impact of L_3 *N. americanus* infective larvae on DC phenotype and function.

iii. The effects of L_3 *N. americanus* infective larvae on the functional properties of epidermal keratinocytes.

iv. Using a 3 dimensional tri-culture of keratinocytes, dermal fibroblasts and DCs at air liquid interface, as a platform for Necatoriasis disease modelling

Additionally, an overview for each chapters is provided, which will be briefly reiterated as an introduction for each chapter respectively.

The primary objective of Chapter 3 was to document the physical interactions between the larvae and DCs, in addition to investigating the phenotype and function of these pivotal immune cells following *N. americanus* infection. In this

chapter, the biological relevance of the glycosylated larvae sheath was examined in the context of interaction with CLRs on DCs. Furthermore, the immunological advantage and role of the *N. americanus* larvae sheaths, in bypassing immune cells at the site of infection was also studied.

In Chapter 4, the purpose of the work was to elucidate the physical interactions between the larvae and epidermal keratinocytes. This chapter examined larval exsheathing and behaviour in culture with epidermal keratinocytes. In conjunction, the morphology, viability and IL-8 cytokine expression of epidermal keratinocytes, following larvae infection was also studied.

The aim of the work described in Chapter 5 was to establish an immunocompetent 3 dimensional tri-culture of keratinocytes, dermal fibroblasts and DCs at air liquid interface, as a platform for Necatoriasis disease modelling. This chapter predominantly examines the development and optimization of an effective skin barrier on cell support scaffolds and platforms. Furthermore, the migration of DCs within the tri-culture in response to potent stimulants was also studied and a Necatoriasis infection model was attempted.

Finally, Chapter 6 encompasses the general conclusions and further work recommendations made for the research presented in this thesis.

CHAPTER 2: Materials and Methods

2.1 Reagents

Minimum essential medium eagle, RPMI 1640 culture media, Dulbecco's modified eagles medium, foetal bovine serum, albumin solution from bovine serum, histopaque, sodium azide, saponin, L-glutamine, penicillin/streptomycin, antibiotic antimycotic solution 100X, non-essential amino acid solution, trypsin-EDTA, phosphate buffered saline, EDTA, paraformaldehyde (PAF), triton X-100, goat serum, fluoromount media, lipopolysaccharide (LPS) derived from *Escherichia coli*, thermoplastic polymer (PET), trifluoroacetic acid (TFA), DC-SIGN (H-200), MR (15.2) and mannan were all purchased from Sigma-Aldrich, UK. FluorSave anti fade medium was purchased from Calbiochem and dichloromethane (DMC) from ThermoFisher Scientific. Standard ELISA development kits for IL-1β (200-01B), TNFα (900-K25), IL-10 (900-K21), IL-12 (900-K96) and IL-6 (900-K16) were purchased from PeproTech. IL-8 (DY208) ELISA kit and cytokine TGF-β (Hek293) from R&D Systems. ZO-1 rabbit polyclonal unconjugated primary antibody (#18-7430) and nuclear stain Hoechst 33342 were purchased from Invitrogen. Secondary antibody ZO-1 Alexa Fluor® 488 goat anti-rabbit IgG (#A27034) and Wheat Germ Agglutinin Alexa Fluor® 488 conjugate from Life Technologies. IL-4, GM-CSF and MACS™ anti-CD14 microbead separation kit from Milteny Biotech. Dendritic cell phenotype antibody markers: anti-CD14 FITC, clone Tuk4; anti-HLA-DR PE-Cyanine5, clone TU36; anti-CD40 PE, clone 5C3 were purchased from ThermoFisher Scientific and anti-CD86 PE, clone IT2.2; anti-CD83 FITC, clone HB15e; Anti-CD206 (MR) PCy5, clone 19.2; anti-CD209

(DC-SIGN) PE, clone eB-h209; anti-CD80 FITC, clone 2D10.4; anti-CD11c PE-Cyanine5, clone 3.9 from eBioscience™. Isotype controls: IgG_1 FITC, clone 2T8-2F5 ; IgG_1 PE, clone 2T8-2F5; IgG_1 PCy5, clone 679.1Mc7; IgG_2a FITC, clone 7T4-1F5; IgG_2b FITC, clone MPC-11; IgG_2b PE, clone MPC-11 and ANNEXIN V– FITC Kit-Apoptosis Detection Kit were all purchased from Beckman Coulter Inc. Normal rabbit IgG (sc-2027) and normal mouse IgG_1 (sc-2025) were purchased from Santa Cruz Biotechnology. Immortalized human epidermal keratinocyte (HaCat) cells were obtained from the Cancer Research Centre, Heidelberg, Germany and immortalized human dermal fibroblast (BJ-6) cells were purchased from ATCC. Dendritic cells were obtained from human blood buffy coats after obtaining written informed consent and approval of local Ethics Committee (National Blood Transfusion Service, U.K.). The hookworms used in this thesis are axenised *N. americanus* L_3 ensheathed larvae and were prepared by Gary Telford (School of Pharmacy, University of Nottingham).

2.2 Methods

The research conducted was a collaborative study in parasitology and immunology, employing methodology which overlaps throughout. To enable the reader better clarification and understanding of the methods utilised, these are segregated into their respective fields.

2.2.1 Immunology

2.2.1.1 Dendritic cell generation

Monocyte derived DCs were generated from peripheral blood monocytes (PBMC's) which were isolated from human blood buffy coats after obtaining written informed consent and approval of local Ethics Committee (National Blood Transfusion Service, U.K.). The PBMC's were isolated via histopaque density gradient centrifugation (Thermo Centrifuge), where 20 ml buffy coat (diluted with phosphate buffered saline (PBS)) was carefully layered onto 15 ml of histopaque and centrifuged at acceleration/deceleration = 1, 800 G, 30 minutes, room temperature. Subsequent to the centrifugation, the PBMC layer was collected, re-suspended in 50 ml PBS and centrifuged twice at acceleration/deceleration = 3, 300 G (1^{st} spin) and 200 G (2^{nd} spin), 10 minutes

each at room temperature. This was repeated again at 200 G with a final re-suspension of PBMCs in 10 ml PBS.

Monocytes were isolated out from the PBMC by incubating the suspension with CD14+ magnetic beads (15 minutes, 4 ^{0}C) and loading it onto a magnetic column. The CD14+ cells remained in the column and were subsequently washed out with MACS buffer (0.5% albumin solution from bovine serum, 2 μM EDTA in PBS) by removing the magnet from the column and flushing through, obtaining a purity of > 98% as described previously (Garcia-Nieto, Johal et al. 2010). Subsequently, purified CD14+ monocytes were cultured with RPMI medium (10% foetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin, and 100 mg/ml streptomycin) supplemented with 50 ng/ml GM-CSF and 250 IU/ml IL-4 in a 24 well tissue culture treated plate (Corning). The cells were cultured for a period of 6 days in standard tissue culture conditions (37 ^{0}C, 5% CO_2) to generate immature DCs (Salazar, Hall et al. 2016).

2.2.1.2 Staining for cell surface markers

In order to prepare the DCs for phenotype analysis via flow cytometer, the cells were harvested and washed twice via centrifugation (acceleration/deceleration = 3, 350 G, 5 minutes, room temperature) in PBA buffer (5% albumin solution from bovine serum, 0.1% Sodium azide in PBS). The supernatant was discarded and the desired antibodies (i.e., CD11c, CD14 and CD83) were added to the

pellet, vortexed and incubated for 20 minutes, dark conditions at 4 ^{0}C. Nonreactive isotype-matched antibodies or unstained cells were used to determine non-specific staining. The samples were then washed with PBA and finally fixed with paraformaldehyde (PAF) solution (0.5% in PBS). This was stored at 4 ^{0}C to be analysed within a 7 day period. The expression of cell surface markers was verified via flow cytometer analysis (Cytomics FC 500, Beckman Coulter) with a minimum of 20,000 events per sample. The data obtained were analysed using the Weasel V.2.7.4 software. Median fluorescence intensity and percentage of positive cells for each marker was determined and further evaluated using GraphPad Prism 6 analysis software.

2.2.1.3 Propagation of epidermal keratinocytes (HaCat) and dermal fibroblasts (BJ-6)

Immortalized human epidermal keratinocytes (HaCat) derived from adult skin and dermal fibroblasts (BJ-6) from new born skin, were cultured and grown in Dulbecco's modified eagles medium (DMEM) and Minimum essential eagle medium (MEM), respectively. The culture media was supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 1% Penicillin/Streptomycin and 1% non-essential amino acid solution. The cells were grown to a confluent layer in a tissue culture treated T75 flask (Corning) at 37 ^{0}C, 5% CO_2.

2.2.1.4 Cytokine expression

The levels of cytokines were measured with ELISA Development Kits (by Helle Sadam, Protobios, Estonia) and were analysed in duplicates, according to the manufacturer's protocol. Absorbance was measured at 450 nm with SpectraMax Paradigm. IL-1β (200-01B), TNFα (900-K25), IL-10 (900-K21), IL-12 (900-K96) and IL-6 (900-K16) were purchased from PeproTech and IL-8 (DY208) from R&D Systems.

2.2.1.5 Viability Analysis

The viability of DCs and HaCat cells were analysed using the Annexin V FITC apoptosis detection kit (Beckman Coulter) according to the manufacturer's protocol. The cells were harvested and washed in ice-cold PBS via centrifugation (acceleration/deceleration = 3, 350 G, 5 minutes, room temperature) and the supernatant discarded. The cell pellet was re-suspended in ice-cold 1x binding buffer, provided in the kit, to a final concentration of $5x10^6$/ml, continuously ensuring the samples were kept on ice. Subsequently, 1 µl of Annexin V FITC solution and 5 µl of propidium iodide were added to 100 µl of the cell suspension re-suspended in binding buffer. This was mixed gently and kept on ice in dark conditions for 15 minutes. Finally, 400 µl of ice-cold 1x binding buffer was added to the samples before being analysed within 30 minutes. The level of Annexin V and propidium iodide that permeated the cells was verified via flow

cytometer analysis (Cytomics FC 500, Beckman Coulter) with a minimum of 20,000 events per sample. In parallel, as a control cell samples were pre-treated with 3% PAF (in PBS) and stained for Annexin V FITC and propidium iodide.

2.2.1.6 Co-culturing epidermal keratinocytes (HaCat) and dermal fibroblasts (BJ-6)

The HaCat cells were cultured as single and co-culture models, (a) on 12 mm transwell plates with polycarbonate membrane inserts (0.4 μm pore size); (b) on a 3 dimensional poly(ethylene terephthalate) (PET) scaffold. The cells were cultured on different platforms in order to measure transepithelial electrical resistance (TEER) values and examine ECM and tight junction protein development.

2.2.1.6.1 Epidermal keratinocytes (HaCat) transwell single culture

The culture media was aspirated from a confluent T75 tissue culture treated flask (Corning) and the HaCat cells were subsequently washed twice with PBS (37 ^{0}C). Trypsin-EDTA was diluted to a final concentration of 0.125% in PBS, and approximately 5 ml was incubated with the cells for 15 minutes (37 ^{0}C, 5% CO_2) to detach the adherent HaCat cells from the tissue culture flask. The cells were then washed twice (acceleration/deceleration = 3, 350 G, 5 minutes, room temperature) in complete DMEM media to remove the trypsin. The cells were

then re-suspended in DMEM media to a density of $1x10^6$/ml, of which 300 µl were seeded onto the apical surface of the polycarbonate membrane inserts (0.4 µm pore size), in the 12 mm transwell plate (Scientific Lab Supplies). Continuing, 500 µl of pre-heated (37 ^{0}C) DMEM was added to the lower chamber. The HaCat cells were grown to confluency while submerged for a period of 72 hours. The culture media was then aspirated from the apical surface, which served to raise the cells to an air liquid interface (ALI) (illustrated in Figure 12).

2.2.1.6.2 Epidermal keratinocytes (HaCat) and dermal fibroblasts (BJ-6) transwell co-culture

In the polycarbonate transwell co-culture model, the HaCat cells were seeded as described, with the substitution of a co-culture media, constituting of a 1:1 DMEM and MEM culture media. The BJ-6 cells were trypsonised and washed as described and re-suspended in the co-culture media to a density of $1x10^6$/ml, of which 300 µl was seeded onto the lower chamber. The cells were grown submerged for a period of 72 hours (37 ^{0}C, 5% CO_2), following which the culture media was aspirated from the apical surface. The schematics of single and co-culture transwell models are illustrated in Figure 12.

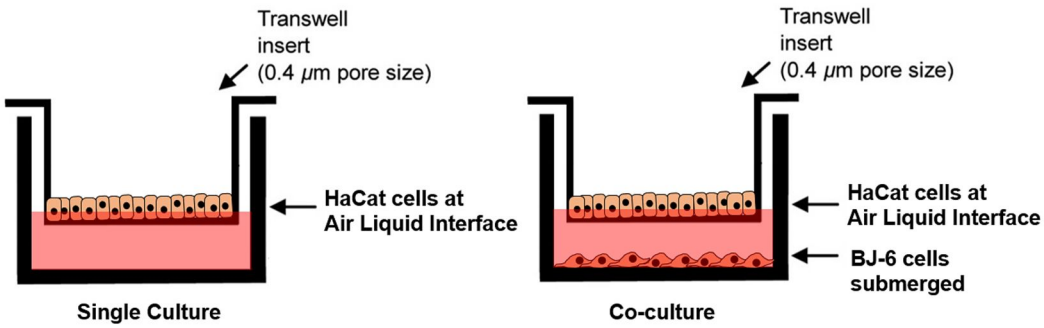


Figure 12: Schematic representation of single and co-cultures of HaCat and BJ-6 cells set up on 12 mm transwell plates with 0.4 µm polycarbonate membrane inserts.

HaCat cells were grown to confluency on the upper layer (transwell insert) and were subsequently raised to ALI in both culture models. The co-culture model differs as it has BJ-6 cells incorporated into the lower chamber and the media constitutes of a 1:1 DMEM and MEM culture media.

2.2.1.6.3 Electrospinning the poly(ethylene terephthalate) (PET) scaffold

PET was dissolved in 1:1 trifluoroacetic acid (TFA) and dichloromethane (DCM) to produce a 10% weight per volume (w/v %) PET polymer solution. The polymer solution was loaded into a syringe (10 ml) with an 18-gauge needle attached (BD Falcon). This was loaded onto a syringe pump-driver (Harvard Apparatus Ltd.) and firmly attached with the tip positioned 15 cm from a steel collector plate. The constant flow rate conditions at which the PET solution was delivered were 0.5 ml/hour at 14 kV for a total period of 4 hours using a high voltage power supply unit (Glassman High Voltage Inc). The process of electrospinning was conducted at room temperature and the collection plate with the fibre mats was placed in a fume hood, allowing evaporation of residual solvents. Finally the electrospun scaffold sheet was removed manually and

stored at room temperature in aluminium foil. The schematics in Figure 13 present a standard horizontal electrospinning setup (Kurusu and Demarquette 2015), which also illustrates the electrospun PET fibres on an SEM micrograph.

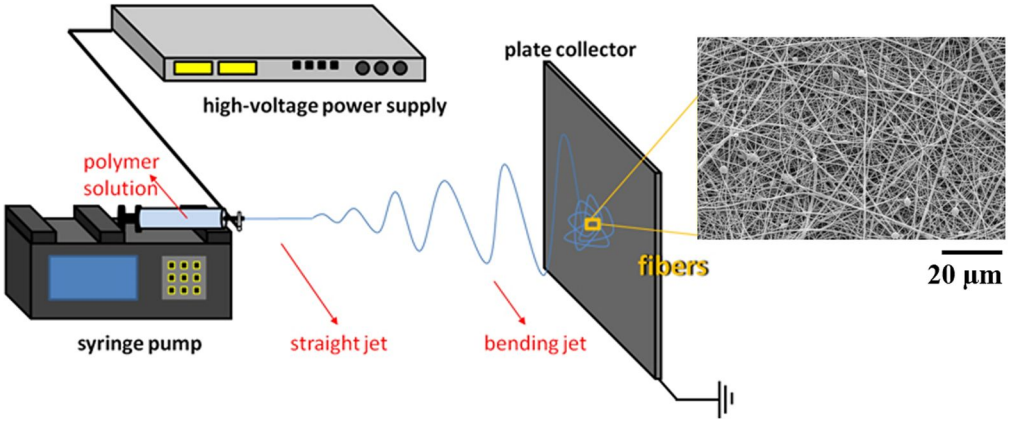


Figure 13: Schematics of a standard horizontal electrospinning setup.

The polymer solution loaded onto a syringe was secured within a syringe pump, with the tip 15 cm from the collector pate. Flow rate at 0.5 ml/hour at 14 kV for a total period of 4 hours and the electrospun PET scaffold is illustrated. Adapted from: (Kurusu and Demarquette 2015).

2.2.1.6.4 Epidermal keratinocytes (HaCat) and dermal fibroblasts (BJ-6) PET scaffold co-culture

The electrospun scaffold was cut into circular pieces with a diameter of 2 cm. These were irradiated and sterilized with ultraviolet (UV) light, 15 min on each side. This was followed by transferring the scaffold fragments to a sterile non-tissue culture treated 12 well plate. Autoclaved steel rings were then placed on top of the scaffold for stabilization, submerging and defining cell seeding area

dimensions. The complete fixation was then further sterilized overnight (37 ^{0}C, 5% CO_2) in antibiotic solution (20% v/v antimycotic/penicillin/streptomycin). Following sterilization, the scaffold was washed twice in PBS (37 ^{0}C) and submerged in the relative cell culture media for 30 minutes (37 ^{0}C, 5% CO_2). The HaCat/BJ-6 were trypsonised as before and seeded at the normal density of 1 x 10^6 cells/ml, of which 300 µl were seeded per steel ring/scaffold. A further 500 µl of cell-free culture media was then added to the external area of the steel ring, and the construct was incubated for a period of 72 hours in standard tissue culture conditions (37 ^{0}C, 5% CO_2). The steel ring was removed and the scaffolds with the seeded cells transferred to a polytetrafluoroethylene (PTFE) platform support (ScaffHolder). This was made in house and designed to support the scaffold at ALI (Harrington, Cato et al. 2014).

The cells were cultured together by placing the HaCat monolayer on top of the BJ-6 monolayer, and were submerged in 1:1 DMEM and MEM culture media for a further 24 hours. The media from the upper chamber was then removed, mimicking the epidermal and dermal layer of the skin where the keratinocytes are at ALI and the fibroblasts submerged (Figure 14). For single HaCat cultures, these were not co-cultured with the BJ-6 monolayer in the Scaffholder and employed only DMEM media.

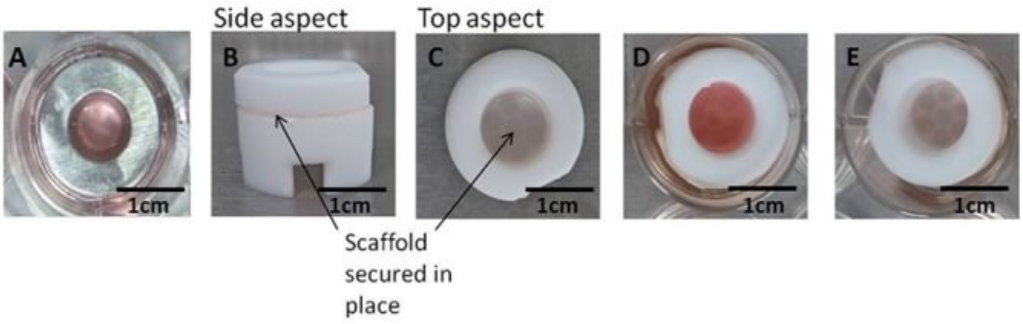


Figure 14: Illustrating the assembly of the bi-layered culture in a Scaffholder.

The steel rings are employed to define the seeding area and submerge the PET scaffold (A). The Scaffholder can hold a single or multiple layers of scaffolds together (B/C). In bi-layered cultures, the HaCat cells are initially submerged (D) however following the aspiration of culture media from the centre of the Scaffholder, the HaCat monolayer is raised to ALI and the BJ-6 cells remain submerged (E). This construct mimics the epidermal and dermal layer of the human skin and can incorporate a further scaffold layer to create a 3D tri-culture construct (Harrington, Cato et al. 2014).

2.2.1.6.5 Epidermal keratinocytes (HaCat) barrier formation in single and co-cultures

Single HaCat cell cultures on transwell inserts were stimulated with TGF-β (2 ng/ml) and GM-CSF (50 ng/ml) diluted in DMEM culture media, for 2 weeks at ALI. In parallel co-cultures of HaCat with BJ-6 cells were cultured at ALI on transwell inserts and PET scaffold. The barrier formation by HaCat cells was characterized through the measurement of TEER, expression of the TJ protein ZO-1 and ECM formation as described previously (Harrington et al. 2014).

2.2.1.6.5.1 Transepithelial electrical resistance measurement

TEER measurements were taken using the STX-2 EVOM voltohmmeter (World Precision Instruments, Inc, UK). For the purpose of TEER analysis, the culture media was preheated (37 ^{0}C) and added to the apical surface of the transwell and Scaffholder. The plate was then placed back into the incubator (37 ^{0}C, 5% CO_2) for approximately 30 minutes. The EVOM voltohmmeter encompasses two electrodes; the external electrode is longer and can be immersed in the lower chamber media, whereas the internal electrode is shorter and is positioned within the apical chamber. These measure the current passing through the transcellular and paracellular pathway of the cells (Srinivasan, Kolli et al. 2015), assessing the barrier function and integrity. The TEER values were recorded every 48 hours with the aspiration of culture media from the apical chamber after measurements to ensure an ALI model (Figure 15).

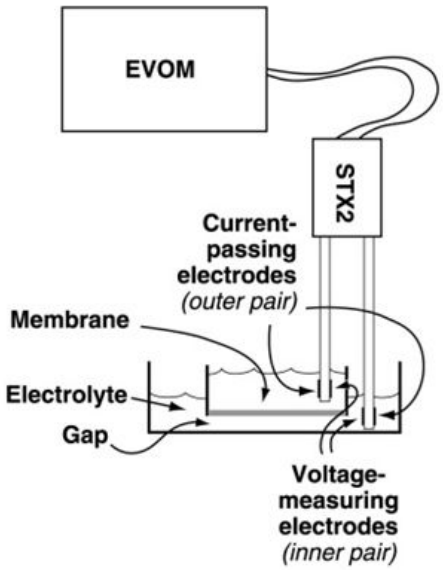


Figure 15: Schematic of measuring TEER using the STX-2 EVOM voltohmmeter.

Culture media is added to the apical surface of the platform and the electrodes are inserted. The internal electrode is positioned within the apical chamber and the longer electrode in the lower chamber, measuring the continuous current passing through the cells via the paracellular and transcellular pathways.

From: https://www.wpiinc.com/blog/2015/11/20/product-information/monitor-the-growth-of-epithelial-cells-in-vitro-manually/

2.2.1.6.5.2 Zonula occludens-1 and Hoechst immunostaining

The samples for ZO-1 staining were washed twice in PBS (room temperature) and fixed overnight (4 ^{0}C) with 4% PAF solution diluted in PBS. This was then washed three times in PBS (3 x 5 minutes each wash) followed by permeabilising the cells with Triton X-100 (0.15% in PBS) for 20 minutes at room temperature. The cells were then blocked for 30 minutes with 5% goat serum diluted in PBS, to prevent nonspecific antibody binding, and washed again in PBS (3 x 5 minutes each wash). The primary antibody (anti ZO-1 mouse monoclonal ab-unconjugated) diluted in 5% goat serum (1:125 dilution) was incubated with the cells for 1 hour at room temperature. The cells were washed again in PBS (3 x 5 minutes each wash) and the secondary fluorescently labelled antibody (ZO-1 Alexa Fluor® 488 goat anti-rabbit IgG) diluted in 5% goat serum (1:200 dilution) was added and incubated at room temperature for 30 min in the dark. The samples were washed again in PBS (3 x 5 minutes each wash) and incubated for 5 minutes with nuclear stain Hoechst 33342 (2 µg/ml) diluted in 5% goat serum under dark condition. The cells were washed for a final time (3 x 5 minutes each wash), embedded in FluorSave medium and mounted onto a glass slide with a cover slip using fluoromount medium. The fluorescently stained images of ZO-

1 and Hoechst were visualized using the Leica SP2 confocal laser scanning microscope and processed via the Leica confocal software (Leica Microsystems Ltd., U.K.).

2.2.1.6.5.3 Scanning electron microscopy

i. ECM evaluation on PET scaffold

Cells were seeded onto PET scaffolds on non-tissue culture treated plates, as described for single and co-cultures of HaCat and BJ-6 cells. The cultures were raised to an ALI for approximately 7 or 14 days prior to analysis. The media was aspirated and the construct was washed twice with PBS before fixing in 4% PAF diluted in PBS, overnight at 4 ^{0}C. The scaffold was then briefly washed with PBS and dehydrated in an increasing Ethanol concentration gradient ranging from 10% - 100%. The dehydrated scaffolds were then mounted onto scanning electron microscopy (SEM) specimen stubs, gold coated and imaged using the standard protocol for SEM.

ii. Dendritic cell interaction with *Necator americanus* on 3D PET scaffold

Immature DCs were generated and re-suspended at a density of 1x10^{6}/ml of which 300 µl were seeded onto a sterilised PET scaffold. The scaffold was pre-treated with RPMI media and submerged by a steel ring in a non-tissue culture treated 12 well plate. A further 500 µl of cell-free RPMI media was then added

to the external area of the steel ring, and the construct was incubated for a period of 24 hours in standard tissue culture conditions (37 ^{0}C, 5% CO_2). Approximately 50 ensheathed L_3 *N. americanus* larvae were re-suspended in 100 µl of RPMI media and added to the submerged PET scaffold with DCs. This was incubated for a further 24 hours at 37 ^{0}C, 5% CO_2. The media was aspirated and the scaffold washed twice with PBS, followed by fixing the construct with 4% PAF overnight at 4 ^{0}C. The scaffold was then briefly washed with PBS and dehydrated in an increasing ethanol concentration gradient ranging from 10% - 100%. The scaffold was mounted onto SEM stubs; gold coated and imaged using standard protocol for SEM.

2.2.1.7 The assembly of an immunocompetent tri-culture

Co-cultures of differentiated HaCat and BJ-6 cells were cultured and assembled on PET scaffolds, held together in a scaffholder at ALI for 14 days. DCs were generated as described previously, and pre-stained with nuclear stain Hoechst 33342 (2 µg/ml) diluted in PBS. These were re-suspended at a density of $1x10^6$/ml of which 300 µl were seeded onto a sterilised PET scaffold. The scaffold was pre-treated with RPMI media and submerged by a steel ring on a non-tissue culture treated 12 well plate. A further 500 µl of cell-free RPMI media was then added to the external area of the steel ring, and the construct was incubated for a period of 24 hours in standard tissue culture conditions (37 ^{0}C, 5% CO_2). The HaCat and BJ-6 construct was cautiously taken apart from the scaffholder, and the DC layer was inserted between these two layers forming a

tri-culture. The now tri-culture construct was replaced back into the scaffholder and the culture media remained at a 1:1 HaCat and BJ-6 culture media (Figure 16).

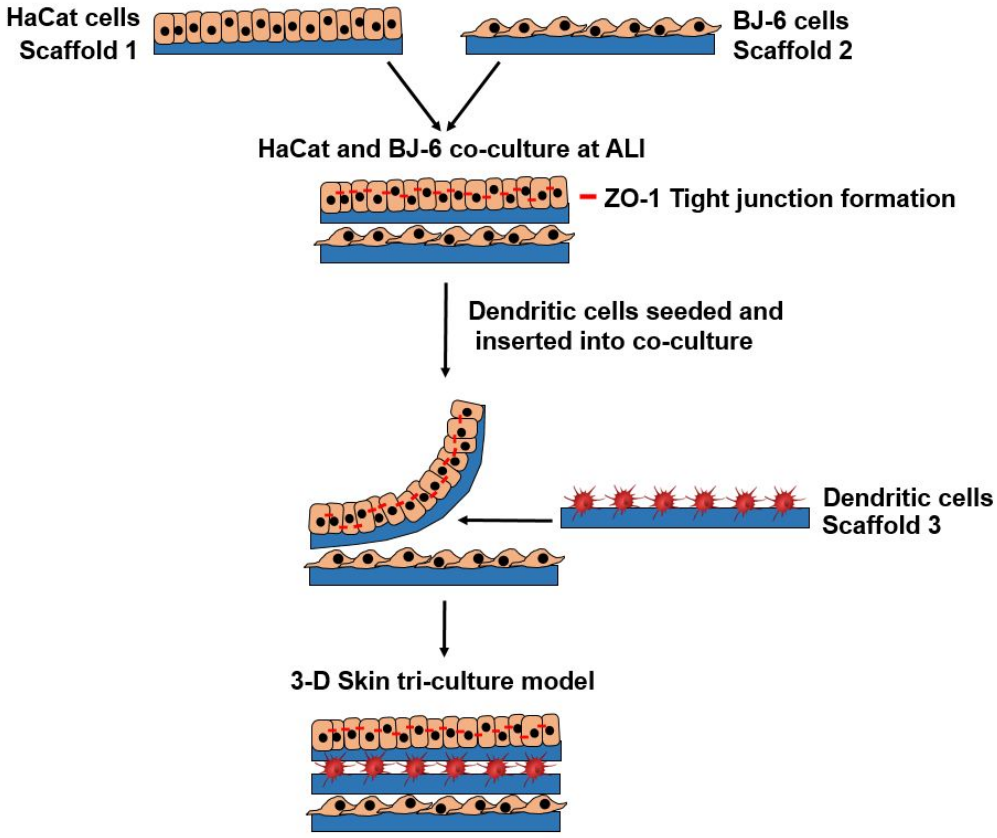


Figure 16: Schematic diagram illustrating the stages of tissue-engineering a 3D immunocompetent *in vitro* model of human skin.

The HaCat cells are seeded onto PET scaffold 1 and BJ-6 onto scaffold 2. These were co-cultured at ALI for 14 days in an in house engineered scaffholder (Harrington, Cato et al. 2014) to enable differentiation and ZO-1 tight junction development. The upper (HaCat) layer is then briefly lifted to insert a separate PET scaffold containing DCs on top of the lower (BJ-6) scaffold layer. The HaCat scaffold layer is then placed on top of the DC scaffold layer, effectively encapsulating this in the middle and forming a tri-culture model.

2.2.1.7.1 Dendritic cell migration

The migration of pre-stained (Hoechst 2 µg/ml) immature DCs was examined in two different tri-cultures; (A) encompassing the HaCat-DC-BJ-6 model and (B) substituting the HaCat and BJ-6 monolayers with acellular scaffolds (i.e., without cells). The constructs were stimulated with 100 ng LPS, 50 ensheathed L_3 *N. americanus* or PBS as a control, for 24 hours (37 ^{0}C, 5% CO_2). The media was aspirated and the construct was fixed with 4% PAF overnight at 4 ^{0}C. The scaffold layers in model (B) were cautiously taken apart and briefly washed with PBS before embedding the samples in FluorSave medium and mounting the monolayers onto a glass slide with a cover slip using fluoromount medium.

In contrast, following overnight fixing in PAF, the scaffold layers in tri-culture model (A) were briefly washed with PBS (3 x 5 minutes each wash) and permeabilized with Triton X-100 (0.15%) for 20 minutes at room temperature. This was followed by 30 minutes incubation in 5% goat serum at room temperature and a further wash in PBS (3 x 5 minutes each wash). The cells were then stained with the primary antibody pan-cytokeratin PK110 (epithelial cell marker) diluted in 5% goat serum (1:100), for 1 hour at room temperature and then washed again in PBS (3 x 5 minutes each wash). The secondary fluorescently labelled antibody (Alexa Flour® 488 goat anti-mouse IgM) diluted in 5% goat serum was added (1:200 dilution), and incubated at room temperature for 30 min in dark conditions. Subsequently, the cells were washed a final time (3 x 5 minutes each wash) and embedded in FluorSave medium and mounted

onto a glass slide with a cover slip using fluoromount medium. The fluorescent images of pan-cytokeratin and Hoechst were visualized using the Leica SP2 confocal laser scanning microscope and processed via the Leica confocal software (Leica Microsystems Ltd., U.K.).

2.2.2 Parasitology

2.2.2.1 Preparation of *Necator americanus* larvae

Infective L_3 *N. americanus* larvae were cultured from faecal material derived from infected individuals as described previously (Kumar, Laouar et al. 1992). This was mixed with water, charcoal and 1% fungizone until a smooth paste was formed. The mixture was applied in a thin layer on the upper half of 0.5 x 8 cm strips of Whatman chromatography paper. The strips were placed in 15 ml centrifuge tubes (containing 2 ml of distilled water) and incubated at room temperature for 8 to 10 days. The water containing the larvae was transferred to a larger cylinder and allowed to settle for 45 minutes. Excess water was aspirated and the concentrated larvae were re-suspended in injection grade water. Larval cultures exceeding >98% motility were used. The larvae were deemed to be axenised following microbiological analysis (FDAS, BioCity, Nottingham).

2.2.2.2 Epidermal keratinocytes (HaCat) incubation with *Necator americanus*

Epidermal keratinocytes (HaCat) were trypsonised and seeded as described, onto a 12 well tissue culture treated plate (Corning), or the apical chamber of the polycarbonate membrane insert (0.4 µm pore size) (Scientific Lab Supplies). The cells were grown to confluency while submerged for a period of 72 hours. The culture media was only aspirated from the apical surface of the transwell insert, which served to raise the cells to an air liquid interface (ALI). Approximately 50 ensheathed L_3 *N. americanus* larvae were re-suspended in 100 µl of DMEM media and were incubated with the HaCat cells on the tissue culture plate and transwell insert, for a further 72 hours (37 ^{0}C, 5% CO_2). Supernatant samples of 20 µl were collected (and replaced with culture media), at 24 and 72 hours. The interactions were imaged throughout (Olympus CKX41, Olympus America) and analysed (Lumenera Infinity Capture software).

2.2.2.3 Dendritic cell incubation with *Necator americanus*

Immature DCs were generated and cultured in a 24 well tissue culture treated plate. Approximately 50 ensheathed L_3 *N. americanus*, re-suspended in 100 µl of RPMI media were incubated with the immature DCs for 24 hours (37 ^{0}C, 5% CO_2). The samples were imaged throughout the incubation period using the ZOE™ Fluorescent Cell Imager (Biorad).

2.2.2.4 C type lectin receptor blocking of dendritic cells

Immature DCs were treated with blocking antibodies, DC-SIGN (H-200), MR (15.2) at 20 µg/ml, or Mannan at 100µg/ml, for 25 minutes at 37 ^{0}C, 5% CO_2 in a 24 well tissue culture treated plate. Subsequently, 50 L_3 *N. americanus* larvae were added to the antibody treated DC culture. The cells were then incubated for a further 24 hours (37 ^{0}C, 5% CO_2) and the samples were imaged throughout using the ZOE™ Fluorescent Cell Imager (Biorad). This was compared to DCs treated with the appropriate isotype controls (rabbit IgG and normal mouse IgG_1) and DCs which were untreated; both conditions were also incubated with the infective larvae. All antibodies were purchased from Santa Cruz Biotechnology.

2.2.2.5 *Necator americanus* incubation with conditioned dendritic cell media

Immature DCs were stimulated with either mannan (100 µg/ml) or LPS (100 ng/ml) for 24 hours in a 24 well tissue culture treated plate, at 37 ^{0}C, 5% CO_2. The cells were centrifuged out (acceleration/deceleration = 3, 350 G, 5 minutes, room temperature) and the cell free conditioned supernatant was incubated with 50 L_3 *N. americanus* larvae for a further hours (37 ^{0}C, 5% CO_2). The samples were imaged throughout using an inverted microscope (Olympus CKX41, Olympus America), and analysed with Lumenera Infinity Capture software. This was compared to the supernatant from untreated DCs, incubated with the infective larvae.

2.2.2.6 *Necator americanus* immunostaining and live imaging

Approximately 50 L_3 *N. americanus* larvae were re-suspended in 1 ml of sterile PBS. This was centrifuged for 5 minutes at acceleration/deceleration = 3, 350 G at room temperature, and the PBS was aspirated. The Wheat Germ Agglutinin (Alexa Fluor® 48 Conjugate) was diluted to a working concentration of 2 µg/ml in PBS, of which 200 µl was incubated with the larvae for 2 hours, dark conditions and at room temperature (Kumar and Pritchard 1992). The larvae were then washed four times (acceleration/deceleration = 3, 350 G, 5 minutes) with 1 ml of sterile PBS. Prior to experimental work i.e., DC-larvae co-culture, the labelled larvae were pre-treated in the appropriate culture media. Samples were imaged using the ZOE™ Fluorescent Cell Imager (Biorad).

2.2.2.7 Mature dendritic cell incubation with *Necator americanus*

Immature DCs were generated and cultured in a 24 well tissue culture treated plate. The cells were stimulated for a period of 24 hours (37 ^{0}C, 5% CO_2) with LPS (100 ng/ml) to stimulate development into mature DCs. The cells were then washed twice in LPS-free RPMI media (acceleration/deceleration = 3, 350 G, 5 minutes) and re-seeded at a density of $3x10^5$ in 500 µl, to which approximately 50 ensheathed L_3 *N. americanus* larvae were added and incubated for a further 24 hours (37 ^{0}C, 5% CO_2). Throughout the incubation period, the samples were imaged using an inverted microscope (Olympus CKX41, Olympus America) and analysed with Lumenera Infinity Capture software.

2.2.2.8 Statistical analysis

The data was analysed and evaluated with the Student t test using GraphPad Prism 6 analysis software. The values of the data are shown as means ±SEM and statistical significance was defined as $p \leq 0.05$, with a correction for multiple comparison employing the Holm-Sidak method.

CHAPTER 3: *Necator americanus* and human derived dendritic cells

3.1 Results

The research presented in this thesis employed viable axenised *Necator americanus* larvae (L_3) provided by the MS clinical trial at the University of Nottingham; a study administering controlled doses of *Necator americanus* for the treatment of MS (Tanasescu and Constantinescu 2015). Due to the nature of this source, the availability of infective larvae for research were inconsistent. Unfortunately, this has prevented further investigation into key observations, and has resulted in several unresolved factors. These are addressed in the discussion, with possible theories and vital avenues, which could be explored for further research.

Albeit this limitation, this chapter describes the work conducted to elucidate the physical interactions between the *N. americanus* larvae and DCs. The phenotype and function of these pivotal immune cells following infection was investigated. In addition, the biological relevance of the glycosylated larvae sheath and its role in bypassing immune cells at the site of infection was also examined.

3.1.1 Characterizing the *Necator americanus* larvae

The *N. americanus* larvae were characterized using an inverted microscope (Olympus CKX41, Olympus America) focusing on their specific hallmark characteristics. The length varied among different larvae, however assessments were made based on the average measurements of 10 larvae, ranging within the standard L_3 hookworm length of 300-400 µm (Figure 17). This was followed by the identification of a non-notched pointed tail with an oesophageal bulb located at a 1/3 of the hookworms length (Figure 18) (Pasuralertsakul and Ngrenngarmlert 2006). These characterizations confirm that the larvae employed in this study are the infective, ensheathed L_3 *N. americanus*.

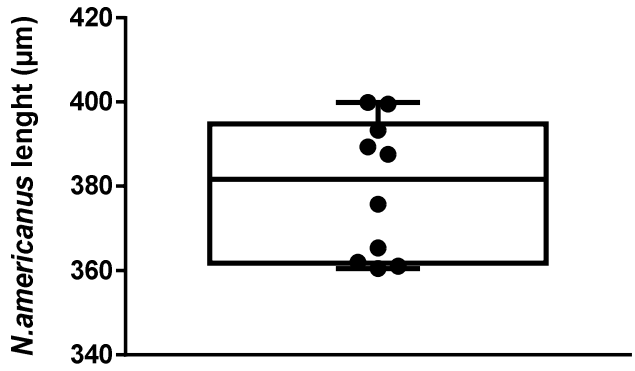


Figure 17: The measurements of L_3 *Necator americanus* larvae length.

The measurements of 10 *N. americanus* larvae were recorded ranging from approximately 360 – 400 µm. Measurements are representative of 3 larvae batches (n=3).

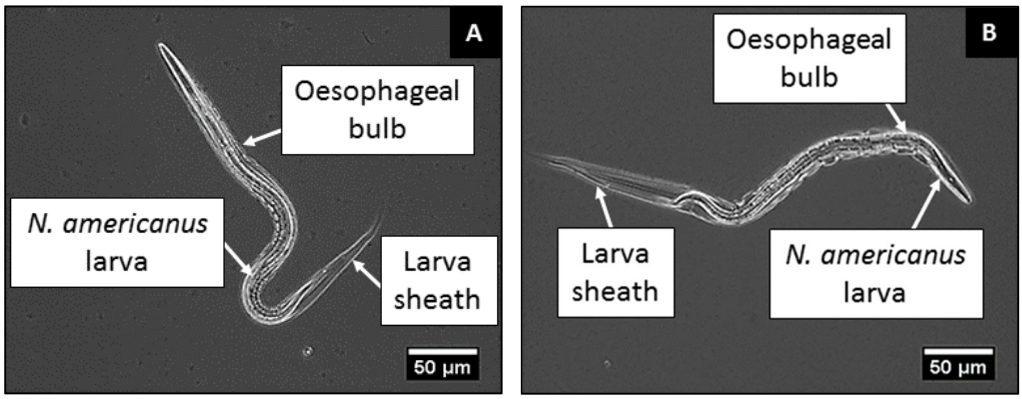


Figure 18: Light microscopy illustrating the hallmark characteristic of L_3 infective *Necator americanus* larvae (Olympus CKX 41, Olympus America).

The L_3 ranges from 300-400 µm with a clustering (bulb) of the oesophagus located approximately at 1/3 along the hookworm's length (A). The hookworm is enclosed in a cuticle sheath and has a distinct pointed non-notched tail (B). Images are representative of 3 larvae batches (n=3).

3.1.2 The physical interaction of *Necator americanus* and immature dendritic cells

The physical interactions and consequences between DCs and *N. americanus* were examined through the incubation of DCs with 50 ensheathed *N. americanus* larvae. The interactions were imaged and monitored using the ZOE™ Fluorescent Cell Imager for up to 24-hours. Microscopy data suggest that DCs bind the larvae sheath, triggering a novel exsheathing process whereby the larvae discard their outer cuticle in response to DCs binding. Exsheathing in this thesis is defined by the initial breach and emergence of the larva from its sheath. The exsheathing occurs at variable intervals which can range up to 4 hours, however no larval exsheathing is observed prior to 1 hour incubation with the DCs. Continuing, DCs are sequestered onto the discarded sheaths, forming aggregates

and do not interact with the exposed larvae. The *N. americanus* migrate away exhibiting negligible interaction with DCs. The exsheathing of a single hookworm was examined, documenting this novel interaction; depicted in a series of snap-shot images highlighting each stage of exsheathing. Additionally, a short film is provided on disc, summarising these interactions (Figures 19/20 and Video 1).

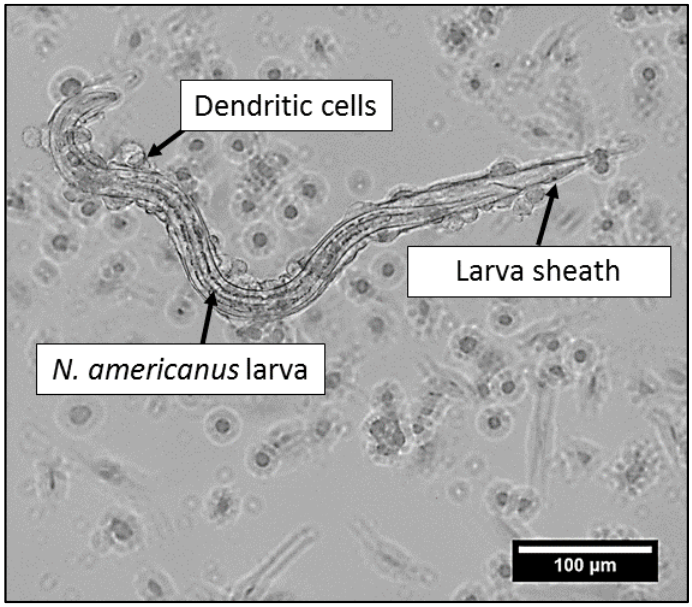


Figure 19: The mechanical interactions of immature dendritic cells with *Necator americanus*.

The sheathed larvae exhibit dynamic movements during the initial incubation period where DCs are sequestered onto the larvae sheath (1 hour). Image representative of 3 larvae batches (n=3).

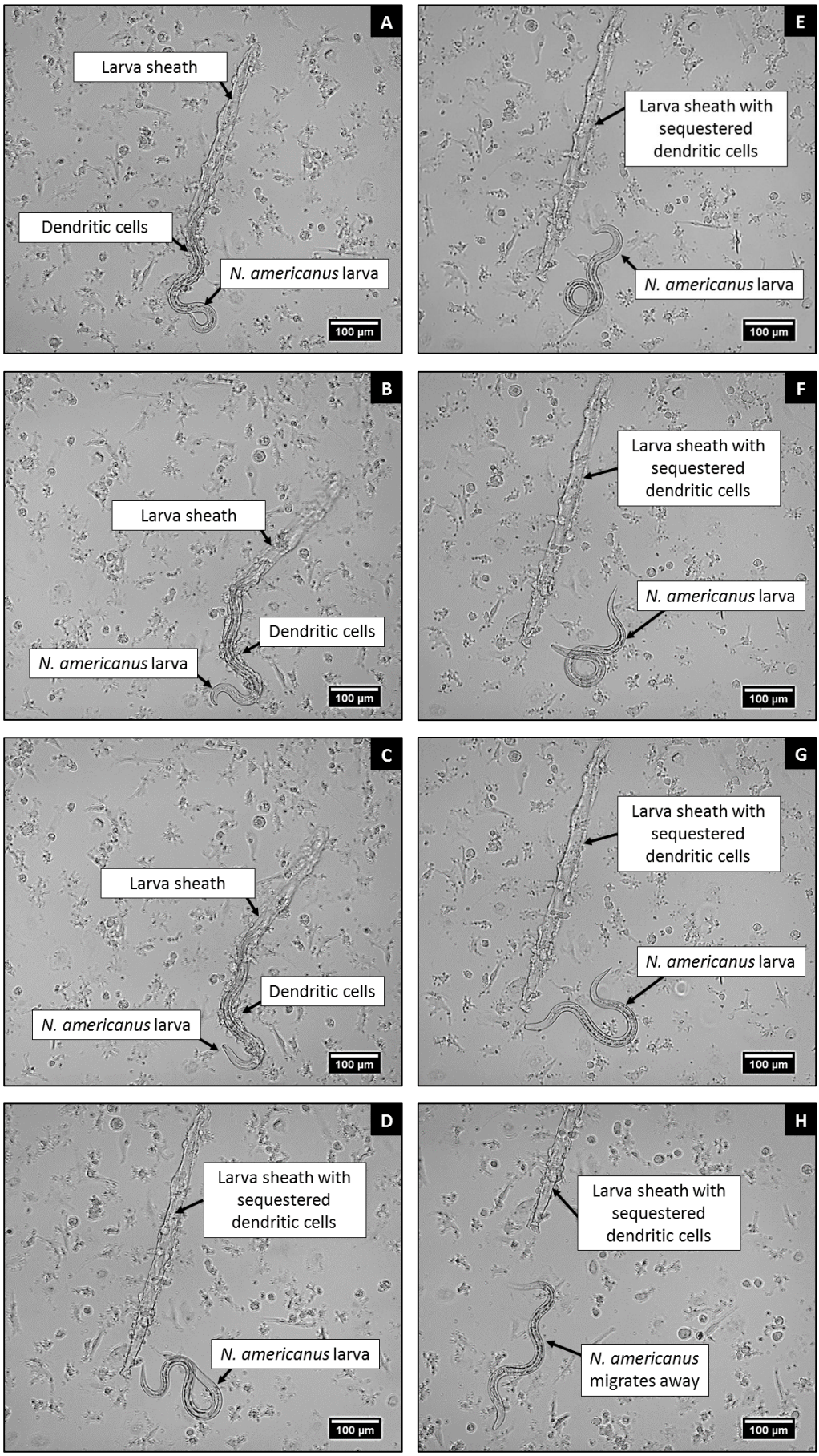

A
Larva sheath
Dendritic cells
N. americanus larva
100 µm
E
Larva sheath with sequestered dendritic cells
N. americanus larva
100 µm
B
Larva sheath
Dendritic cells
N. americanus larva
100 µm
F
Larva sheath with sequestered dendritic cells
N. americanus larva
100 µm
C
Larva sheath
Dendritic cells
N. americanus larva
100 µm
G
Larva sheath with sequestered dendritic cells
N. americanus larva
100 µm
D
Larva sheath with sequestered dendritic cells
N. americanus larva
100 µm
H
Larva sheath with sequestered dendritic cells
N. americanus migrates away
100 µm

Figure 20: The mechanical interactions of immature dendritic cells with *Necator americanus*.

The still ensheathed larva begins to move dynamically until it finally breaches its sheath (A). Once the larva begins exsheathing, the process occurs within seconds as the sheathed hookworm exits the cuticle sheath swiftly. For this individual larva exsheathing was initiated at the 4-hour time point. At the first sight of exsheathing, images were captured sequentially with 10 second intervals (B: 10 seconds; C: 20 seconds). The hookworm progressively discards the sheath (B and C) until it is fully exsheathed (D) and migrates away from its discarded cuticle sheath with sequestered DCs (H). Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

3.1.3 The effects of conditioned dendritic cell media on *Necator americanus* exsheathing

The exsheathing and sequestration of DCs onto the larvae sheath, is a novel finding. To elucidate whether this interaction is due to soluble factors, DCs were stimulated with mannan (100 μg/ml) or LPS (100 ng/ml) and the conditioned, cell free media was collected after 24 hours. The *N. americanus* larvae were then incubated with the conditioned media for 24 hours as described. In parallel, media from unstimulated DCs was collected and incubated with the larvae as a control. Intriguingly, the hookworms do not exsheath in culture with DC-free conditioned media, indicating that a cell mediated interaction is required for the induction of exsheathing. Additionally there was a significant decrease in larvae movement, compared to those in culture with DCs (Figure 21).

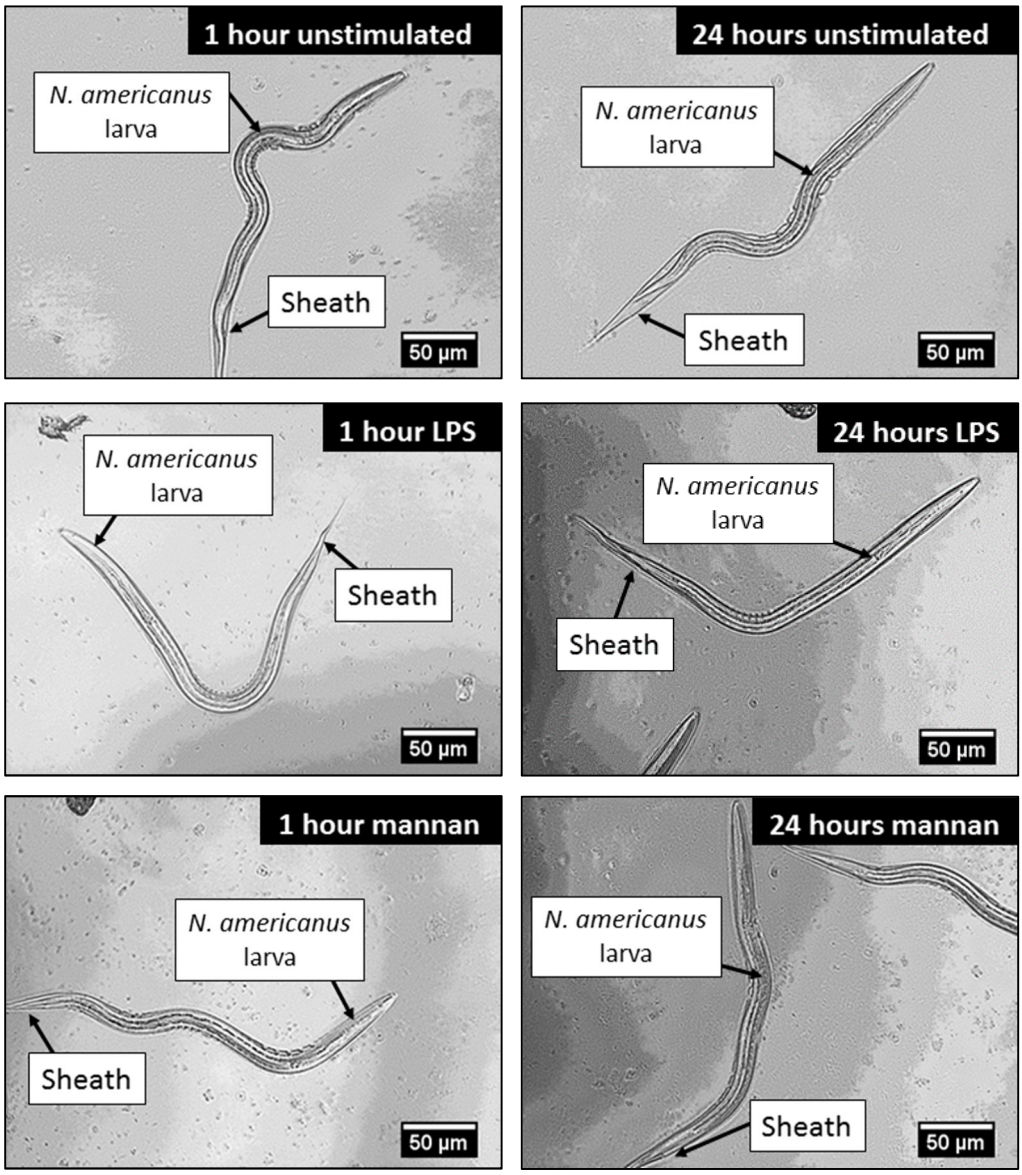


Figure 21: *Necator americanus* larvae treatment with conditioned media from dendritic cells.

N. americanus larvae do not exsheath in culture with cell-free conditioned media from LPS, Mannan or unstimulated DCs. Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

3.1.4 Elucidating the mechanism of dendritic cells and larvae interactions

Previous research has distinguished *N. americanus* from other nematode species employing its distinct lectin binding capabilities (Kumar and Pritchard 1992). It was therefore reasonable to assume that lectins on the hookworm sheath could meditate the interaction with DCs. To investigate this possibility DC-SIGN and MR on DCs were blocked using specific antibodies, or mannan, before incubation with *N. americanus* larvae for 24 hours. The data illustrated a significant decrease in the induction of larvae exsheathing and the sequestration of DCs onto larvae sheaths; in comparison to those observed in the presence of untreated DCs (Figure 22). Notably in untreated conditions, the initial DCs which are sequestered onto the sheath form large aggregates where bystander cells part of the accumulation are not in direct contact with the larvae sheath. This alludes to an adhesion mechanism initiated by the sequestered DCs.

Furthermore, blocking either of these receptors prevents exsheathing all together. This not only indicates a role for MR and DC-SIGN in mediating interactions between DCs and the larvae, but that both CLRs are mandatory for the novel larval exsheathing and DC sequestration observed.

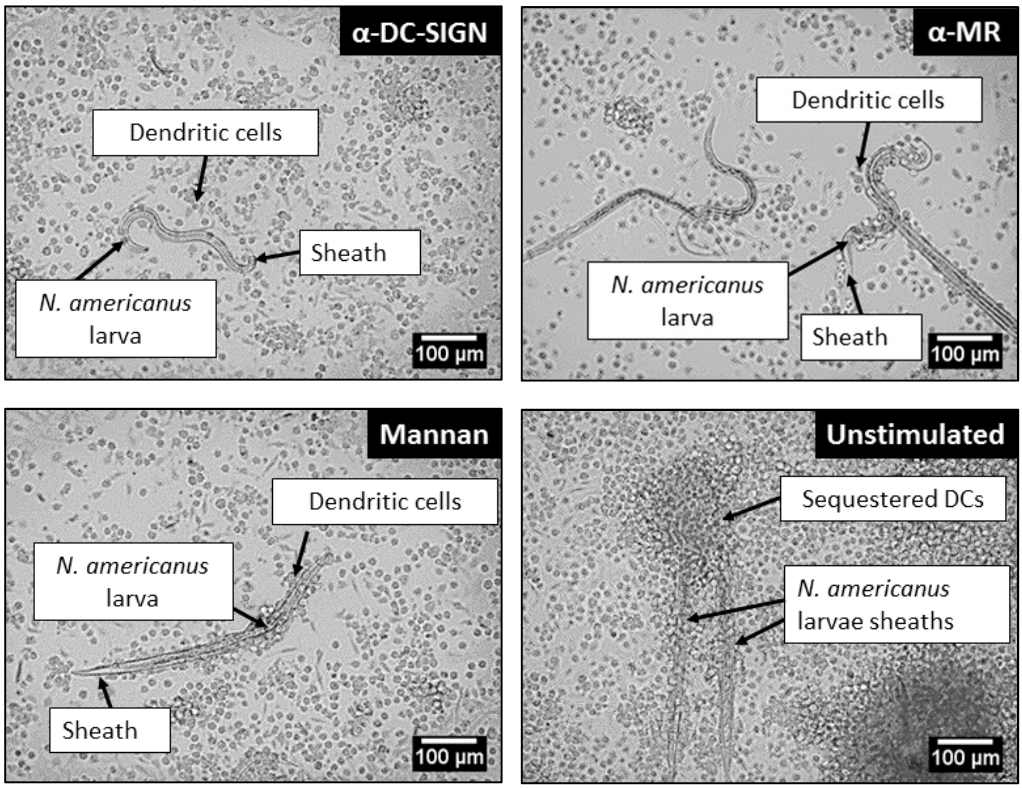


Figure 22: The incubation of *Necator americanus* larvae with α-CLRs or mannan treated dendritic cells.

Following the blocking of CLRs on the surface of DCs, and incubation of larvae for 24 hours, there is a substantial decrease in DCs sequestration and aggregate formation. These larvae also remain ensheathed. In contrast, untreated DCs are sequestered onto the discarded sheath forming large aggregates. Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

3.1.4.1 Prolonged dendritic cell treatment with α-CLRs

As observed, the treatment of DCs with α-CLRs (DC-SIGN and MR) or mannan prevents the excessive exsheathing and sequestration of DCs onto the larvae sheath (Figure 22). Rationally the extent of this effect was investigated through treatment with antibodies or mannan for a prolonged period of time (48 hours). Intriguingly at 48 hours post treatment with α-DC-SIGN, α-MR or mannan, the

inhibition in cell sequestration and aggregate formation is negated (Figure 25). In parallel, DCs treated with the respective isotype control antibodies (rabbit IgG and normal mouse IgG_1) were also examined. There were no changes observed in comparison to untreated DCs and larvae interactions (Figure 24).

Nevertheless, it is fundamental to highlight that untreated DCs are normally spread evenly and localise in the middle of the tissue culture well (2 cm^2 surface area) (Figure 23). However, following 24 hours in culture with the larvae, the media becomes significantly 'cloudy'. This is further exaggerated by DCs accumulating in the areas with *N. americanus* larvae, and forming dense cell aggregates (Figure 25). Consequently, this interfered with the quality of micrographs obtained and identification of larvae sheaths. Therefore, to better visualise the larva sheath within these cell aggregates, they were stained with a fluorescent labelled lectin Wheat Germ Agglutinin (WGA), Alexa Fluor® 488 Conjugate as described previously (Brown, Girod et al. 1999). The interactions were visualized using ZOE™ Fluorescent Cell Imager (Figure 26). Despite highly dense cell aggregates surrounding larvae sheath cuticles, they can still be visualised within some aggregates. However, the DCs were also stained by the WGA lectin dye, which was probably due to the detection of glycosylated lectin ligands present on DCs (El Sherbini, Hock et al. 2000).

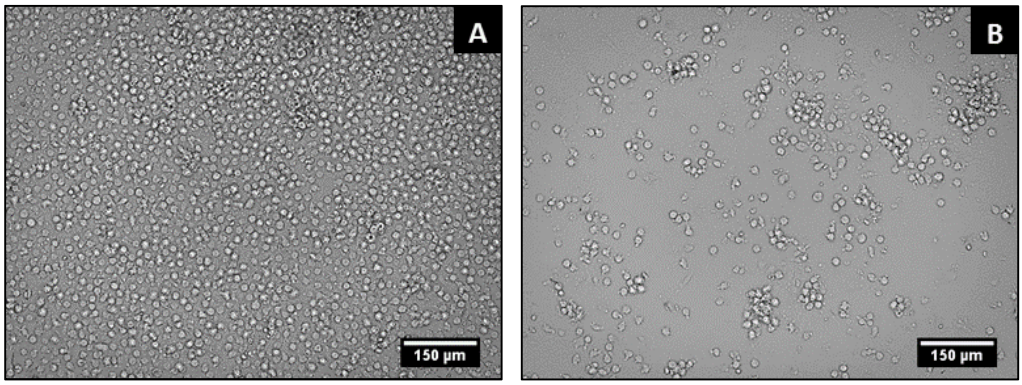


Figure 23: The localization of immature dendritic cells in a 24 well tissue culture plate. (A) Centre (B) Outer edge.

The cells are seeded at a density of $3x10^5$, where they localise towards the central area and are evenly spread. Outside of the centre and towards the outer edges of the well, the density of cells decrease significantly. Images are representative of 3 independent experiments using DCs from 3 different donors (n=3).

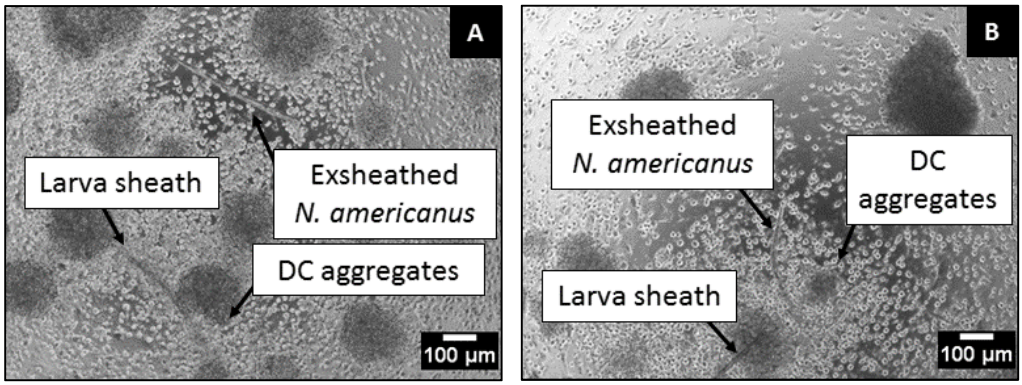


Figure 24: Isotype control treated dendritic cells interaction with *Necator americanus.* Rabbit IgG (A) and normal mouse IgG1 (B).

The DCs were treated with isotype controls for 24 hours and exhibited no inhibition in the sequestration of DCs onto the larvae sheath or forming large adhesion aggregates. Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

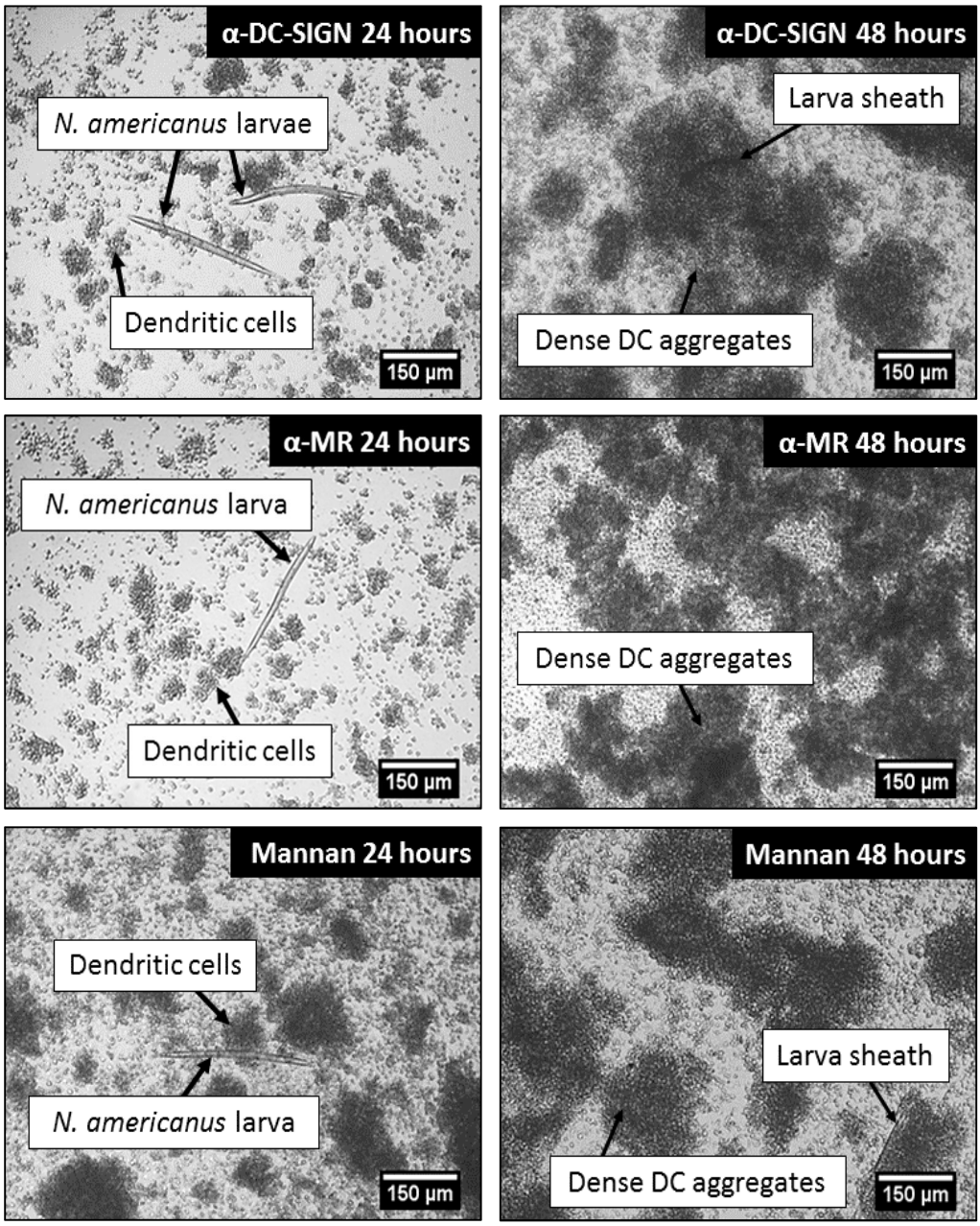


Figure 25: Prolonged blocking treatment of CLRs on dendritic cell surface and *Necator americanus* incubation.

There is a substantial decrease in DC aggregates around *N. americanus* larvae in the presence of α-DC-SIGN, α-MR or mannan at the 24 hour time point. In contrast, at the 48 hour time point, DCs are sequestered and form large dense aggregates. Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

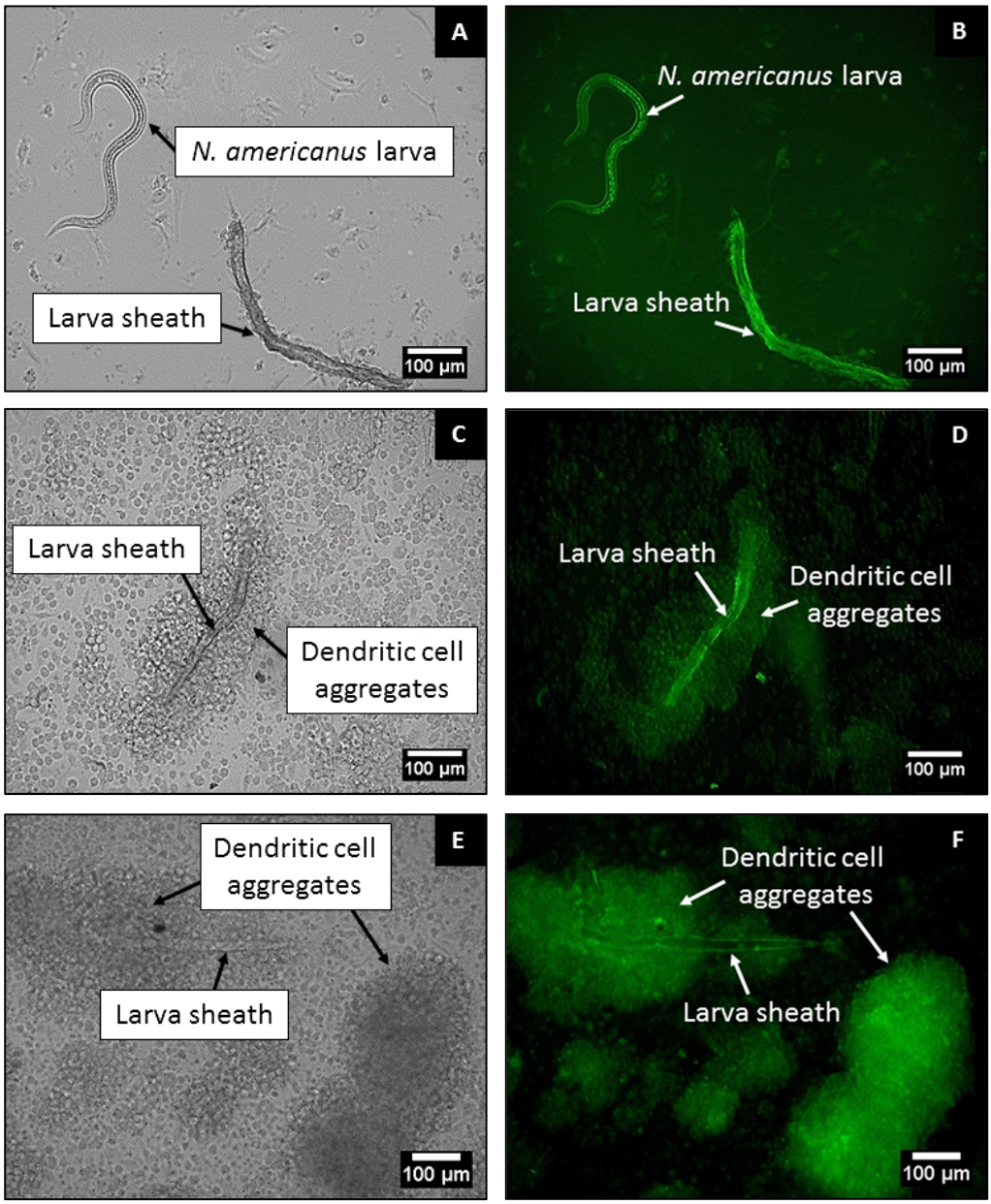


Figure 26: Distinguishing and identifying the *Necator americanus* larvae sheaths in dendritic cell aggregates.

The exsheathed larvae captured at 4-hours post incubation with DCs on light (A) and fluorescent microscopy (B). However at 24-hours the DCs aggregates interfere with the observations and hinder the precise identification of the discarded larvae sheath on light microscopy (C and E). Following close observation, the corresponding fluorescent microscopy data identifies the hookworm sheath to be present amid the DCs aggregates (D and F). Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

3.1.5 The lysis of *Necator americanus* by mature dendritic cells

As illustrated, the cell-free conditioned media from DCs treated with LPS or mannan had no visible effects on larvae viability (Figure 21). Consequently, the effects of mature DCs (in LPS-free media) on *N. americanus* larvae was investigated to elucidate these interaction further. DCs were stimulated for a period of 24 hours with LPS (100 ng/ml) and washed in RPMI media to remove the LPS from culture. Approximately 50 infective L_3 *N. americanus* were incubated with the mature DCs for 24 hours at 37 ^{0}C, 5% CO_2.

At the 1 hour time point, the *N. americanus* larvae remain ensheathed and DCs do not adhere to the cuticle sheath. Additionally, these hookworms exhibit no vigorous movements as observed in the presence of immature DC. Intriguingly, 24 hours post incubation with mature DCs, the larvae are completely lysed characterized by the identification of intracellular remains within the cuticle sheath. The capability of mature DCs to lyse *N. americanus* within its sheath, and consequently rendering it non-viable, is a novel finding. This is illustrated in Figure 27, documenting the lysis of a single hookworm.

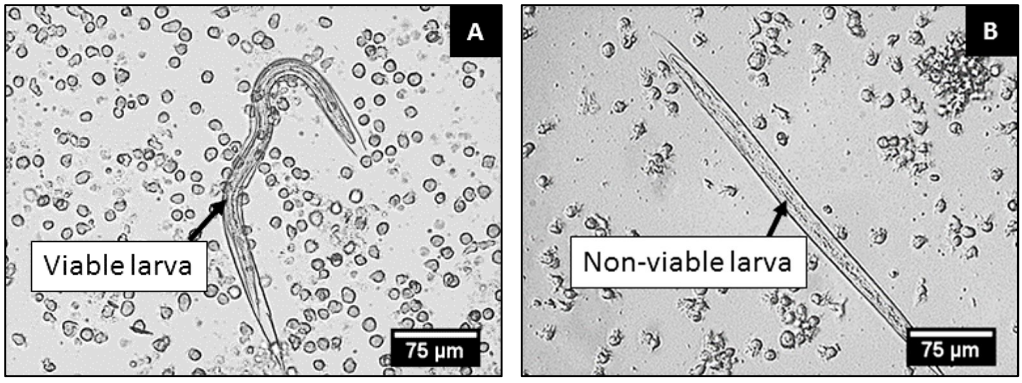


Figure 27: The lysis of *Necator americanus* larvae by LPS matured dendritic cells.

At 1 hour (A) post incubation with mature DCs in LPS-free media the larva is intact, viable and remains ensheathed. In contrast at the 24 hour time point (B), albeit the sheath is visible, the encapsulated larva within is lysed. Images are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

3.1.6 Dendritic cell surface phenotype in response to *Necator americanus*

Primarily, the population of DCs selected were assessed to ensure that the CD14+ monocytes cultured in the presence of GM-CSF and IL-4 media were fully differentiated into CD14- immature DCs. The cells stained positive for CD11c, albeit this marker in humans is found on myeloid DCs and monocytes. However the DCs stained negative for CD14, which is a marker DCs lack (Collin, McGovern et al. 2013). The dot plots for CD14-/CD11c+ DCs is shown illustrating the morphology, size, fluorescence and optical homogeneity of the selected DC population (Figure 28).

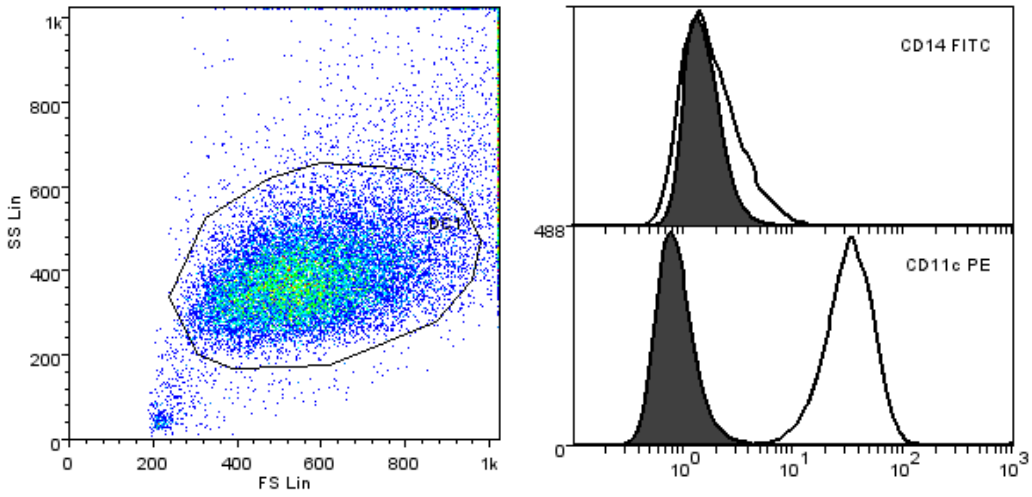


Figure 28: Illustrating the dot plot and histogram of the selected CD14-/CD11c+ dendritic cell population.

In humans both myeloid DCs and monocytes express CD11c, however DCs lack the CD14 receptor. The population of cells selected are CD14-/CD11c+ monocyte derived immature DCs. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

The data presented in this chapter illustrates the DCs binding the larvae cuticle sheath (i.e., Figure 19), signifying antigen capturing capabilities. The recognition of a foreign antigen initiates DCs to go through several phenotypical modifications (Howard, Charleston et al. 2004); rationally the effects of *N. americanus* larvae on DC phenotype and function were assessed. DCs were incubated with *N. americanus* larvae for 24 hours, in parallel DCs were also stimulated with 100 ng/ml LPS to induce maturation. The phenotype of DCs were analysed using FACS as described in the methodology, and the median fluorescence intensity (MFI) for each cell surface marker was measured. The markers investigated were CD80, CD83, CD86, CD40, CD206, CD209, and HLA-DR (n=3).

The findings demonstrate that DCs incubated with the larvae maintained an immature phenotype as evidenced by a lack of up-regulation in maturation markers CD80, CD83, CD86, CD40 and HLA-DR. In addition, there was a significant downregulation in CD206 expression. All phenotype data were normalized by subtraction of median fluorescence intensity from isotype controls i.e., IgG_1 FITC, IgG_1 PE and IgG_1 PCy5 or unstained samples. The experiments were independent, using DCs from 3 different donors and 3 larvae batches (n=3). Additionally the corresponding histograms for all donors are also presented.

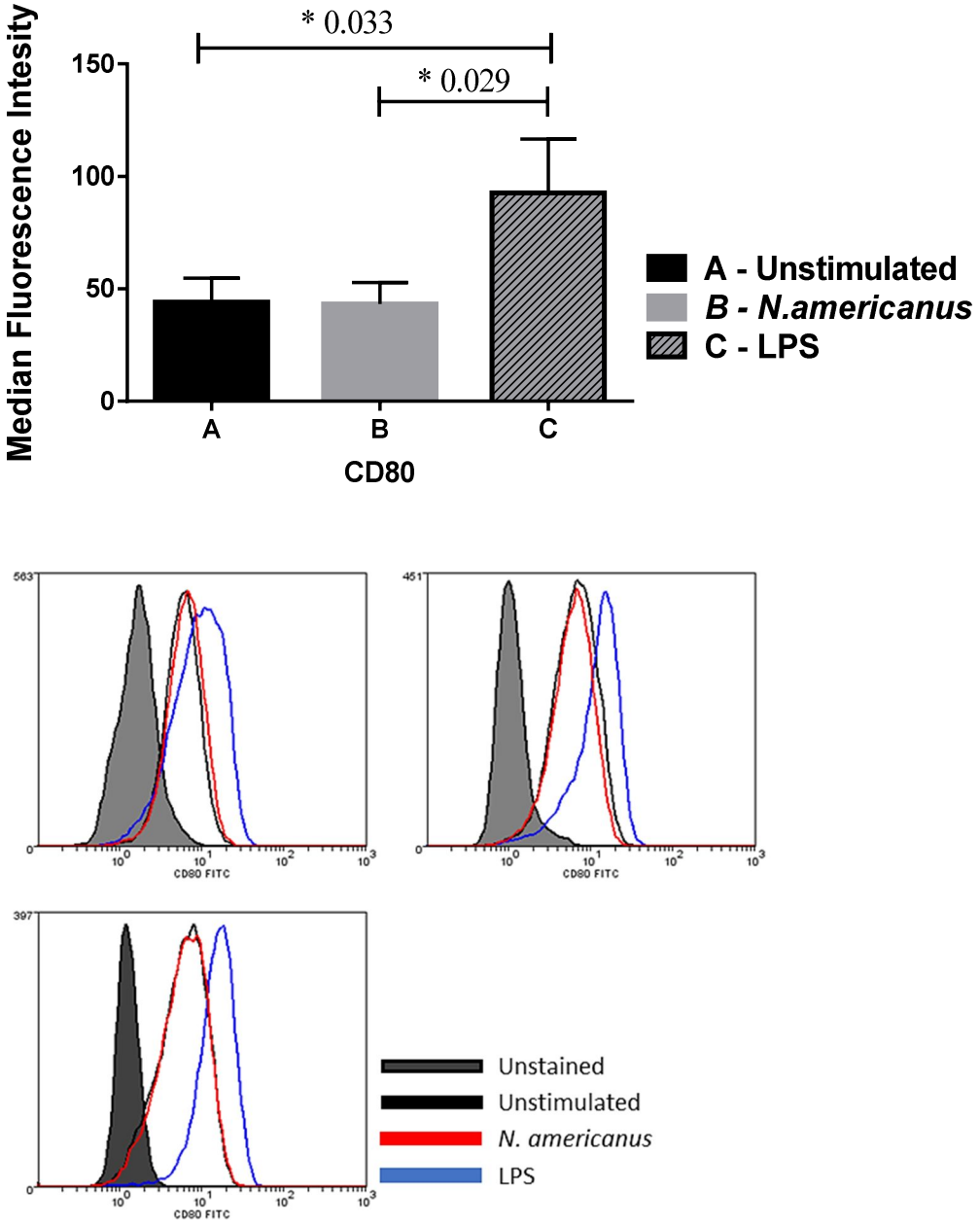


Figure 29: The expression of dendritic cell receptor molecule CD80 in response to stimulation with LPS or *Necator americanus* (± SEM).

In response to LPS stimulation the DCs exhibited a significantly higher expression of the marker molecule CD80 ($p = 0.029$). This is reflected in the histograms for all donors, with a clear shift and increase in the fluorescence detected. In contrasts DCs stimulated with the infective larvae maintained their immature state by exhibiting a lack of up-regulation in the receptor. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

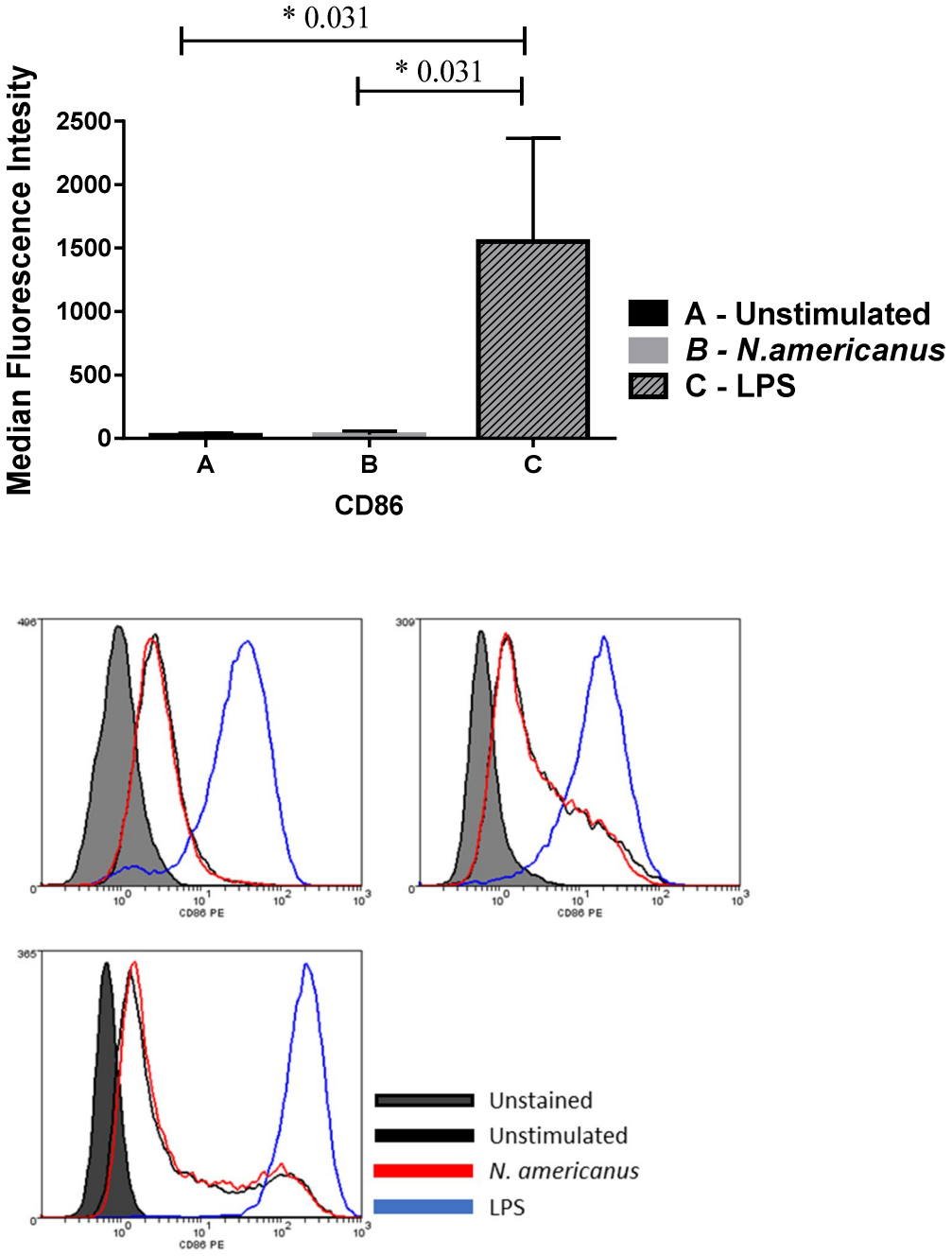


Figure 30: The expression of dendritic cell receptor molecule CD86 in response to stimulation with LPS or *Necator americanus* (± SEM).

In response to LPS stimulation the DCs exhibited a significantly higher expression of the marker molecule CD86 ($p = 0.031$). This is reflected in the histograms for all donors, with a clear shift and increase in the fluorescence detected. In contrasts DCs stimulated with the infective larvae maintained their immature state by exhibiting a lack of up-regulation in the receptor. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

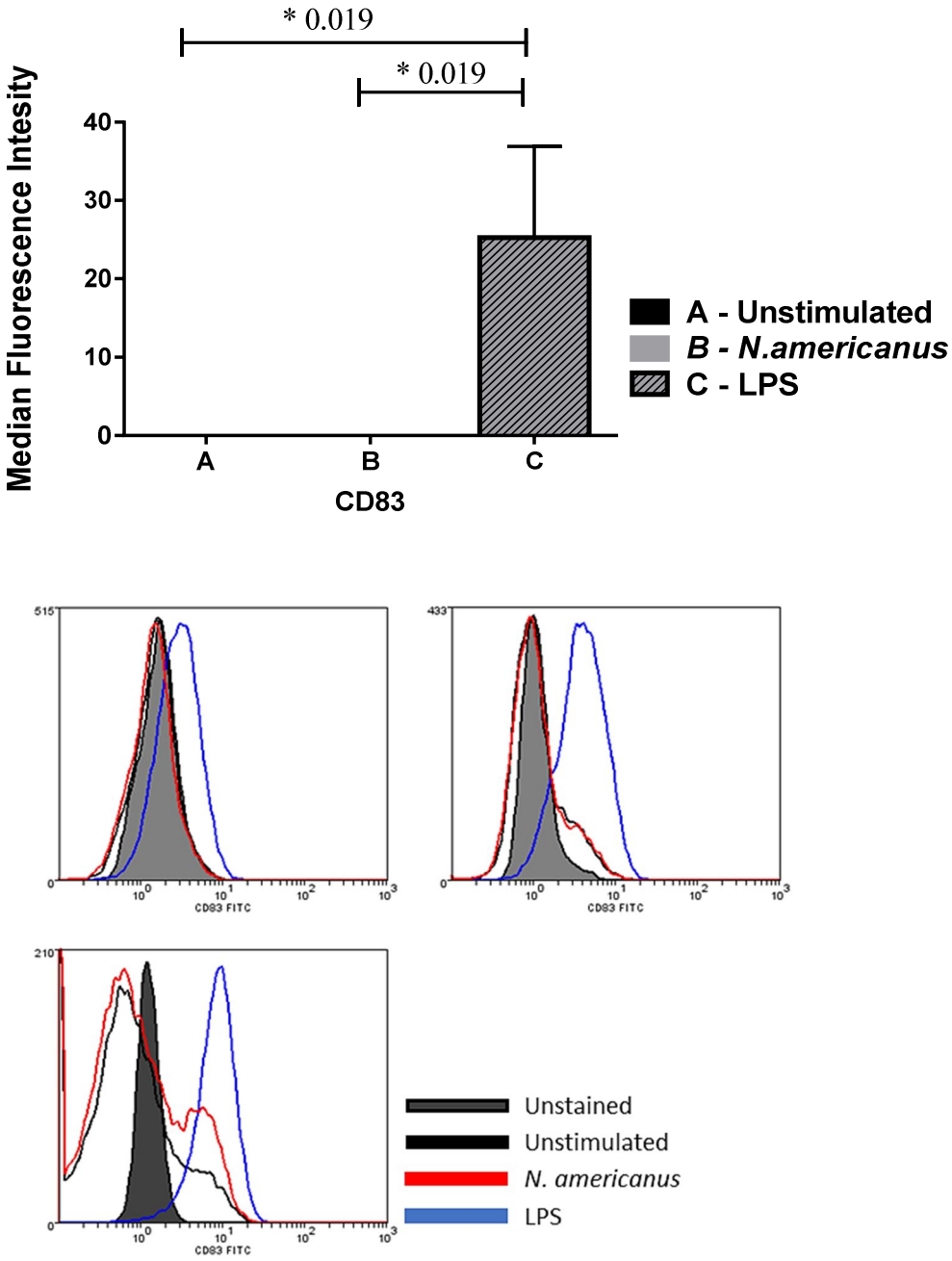


Figure 31: The expression of dendritic cell receptor molecule CD83 in response to stimulation with LPS or *Necator americanus* (± SEM).

In response to LPS stimulation the DCs exhibited a significantly higher expression of the marker molecules CD83 ($p = 0.019$). This is reflected in the histograms for all donors, with a clear shift and increase in the fluorescence detected. In contrasts DCs stimulated with the infective larvae maintained their immature state by exhibiting a lack of up-regulation in the receptor. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

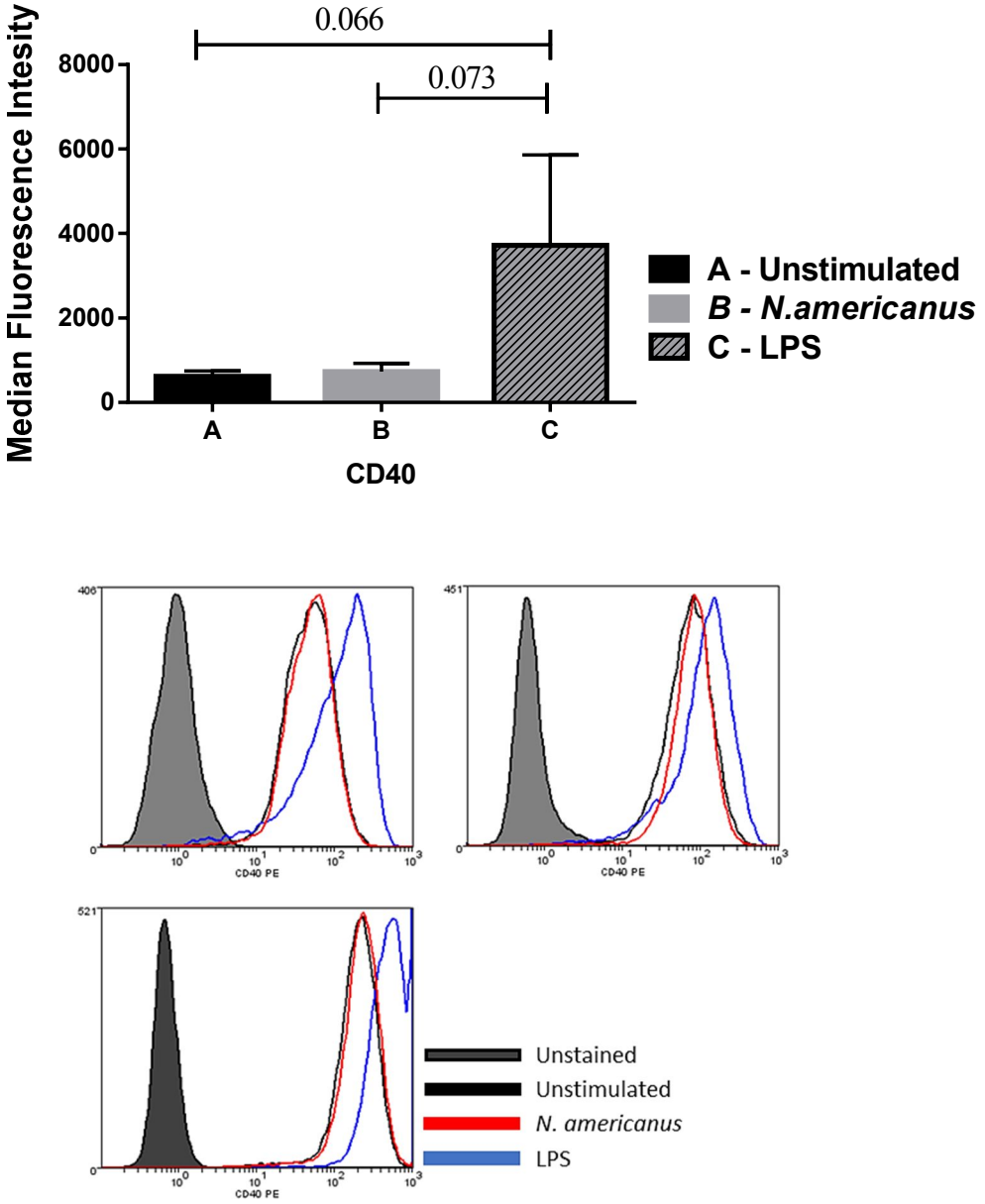


Figure 32: The expression of dendritic cell receptor molecule CD40 in response to stimulation with LPS or *Necator americanus* (± SEM).

In response to LPS stimulation the DCs exhibited a non-significant increase of the marker molecules CD40 ($p = 0.073$). This is reflected in the histograms for all donors, with a small shift and increase in the fluorescence detected. In contrasts DCs stimulated with the infective larvae exhibited a lack of up-regulation in the receptor. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

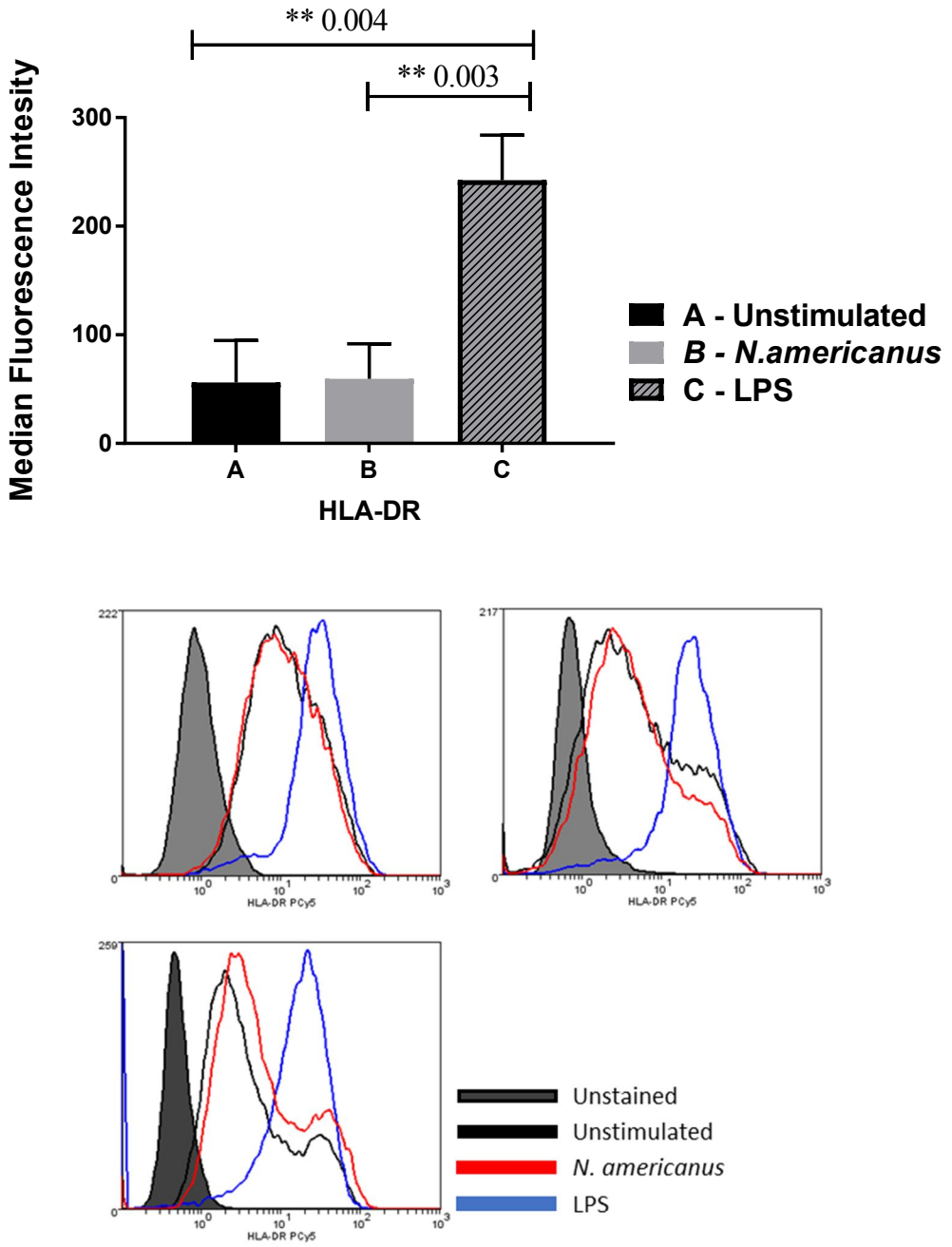


Figure 33: The expression of dendritic cell receptor molecule HLA-DR in response to stimulation with LPS or *Necator americanus* (± SEM).

In response to LPS stimulation the DCs exhibited a significantly higher expression of the marker molecule HLA-DR ($p = 0.003$). This is reflected in the histograms for all donors, with a clear shift and increase in the fluorescence detected. In contrasts DCs stimulated with the infective larvae exhibited a lack of up-regulation in the receptor. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

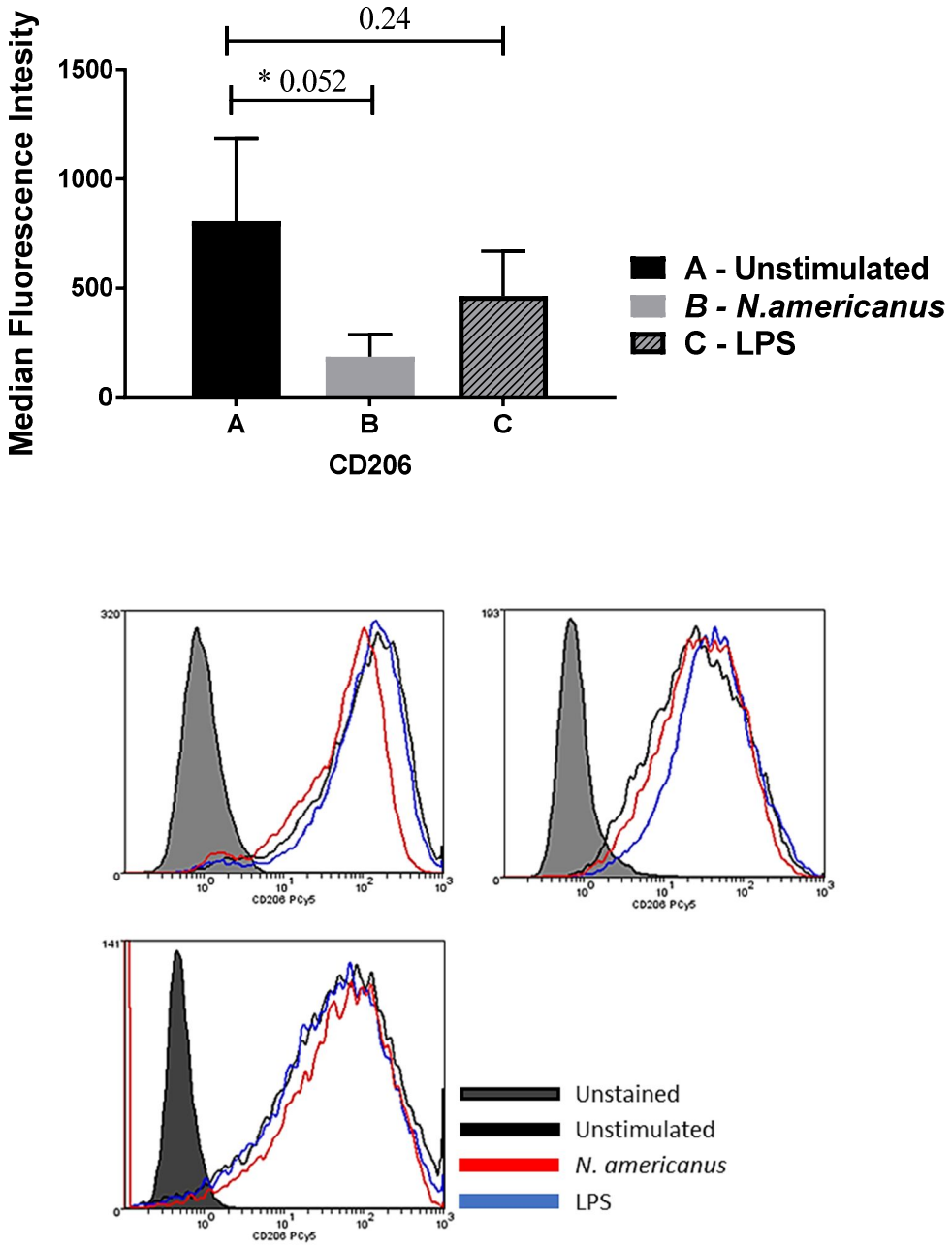


Figure 34: The expression of dendritic cell receptor molecule CD206 in response to stimulation with LPS or *Necator americanus* (± SEM).

DCs stimulated with the infective larvae exhibited a significant decrease in CD206 ($p = 0.052$). However in response to LPS, there was an overall decrease observed, which was varied and more apparent between individual donors as illustrated in the histograms. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

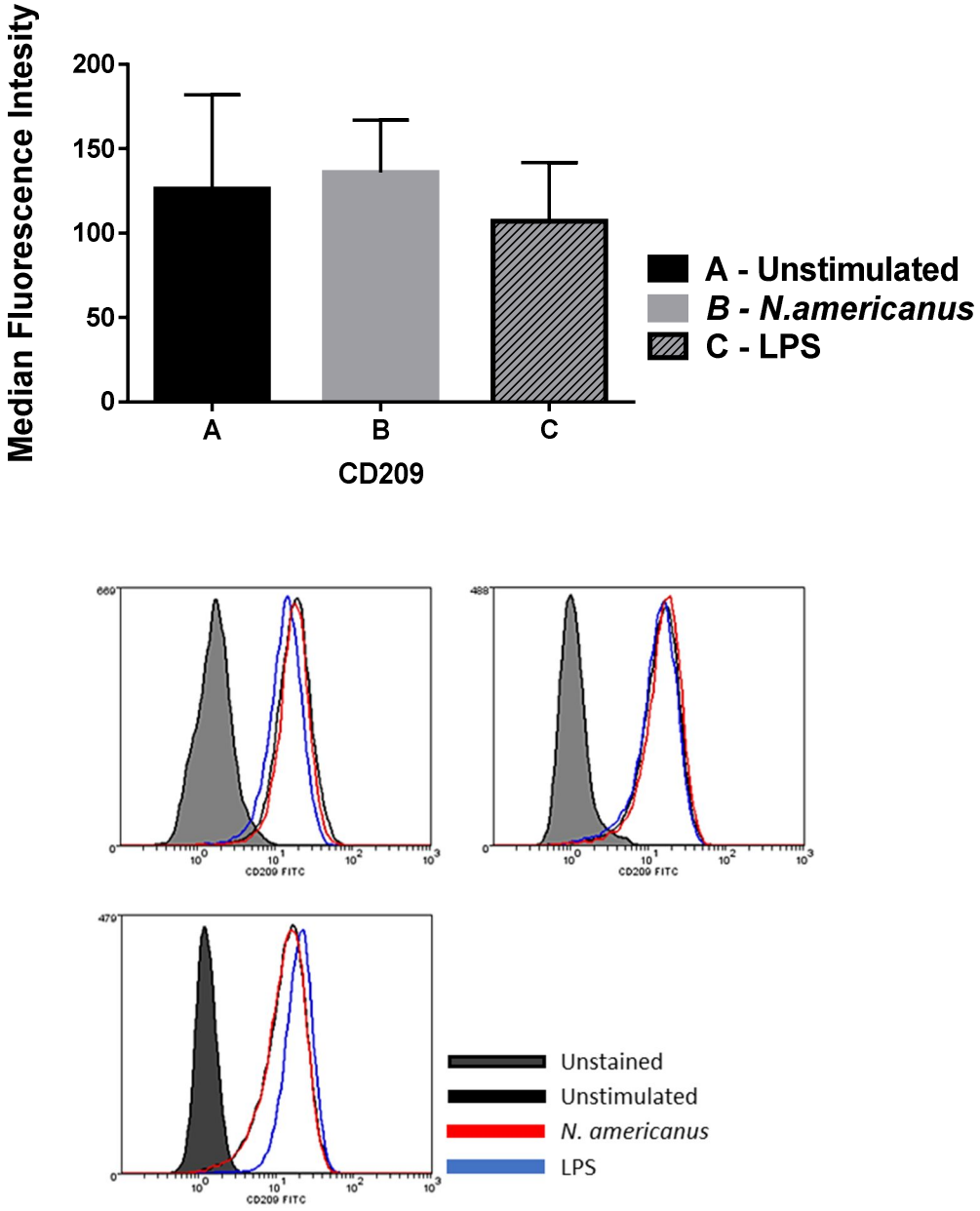


Figure 35: The expression of dendritic cell receptor molecule CD209 in response to stimulation with LPS or *Necator americanus* (± SEM).

DCs stimulated with the infective larvae exhibited no difference in CD209 expression. However, in response to LPS, there was an overall decrease observed, which was varied and more apparent between individual donors as illustrated in the histograms. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

Subsequently DCs ability to remain responsive to a potent stimulant such as LPS, whilst in culture with *N. americanus* was assed. In response to co-stimulatory conditions (LPS + *N. americanus*) the DCs exhibited no impairment in responding to LPS.

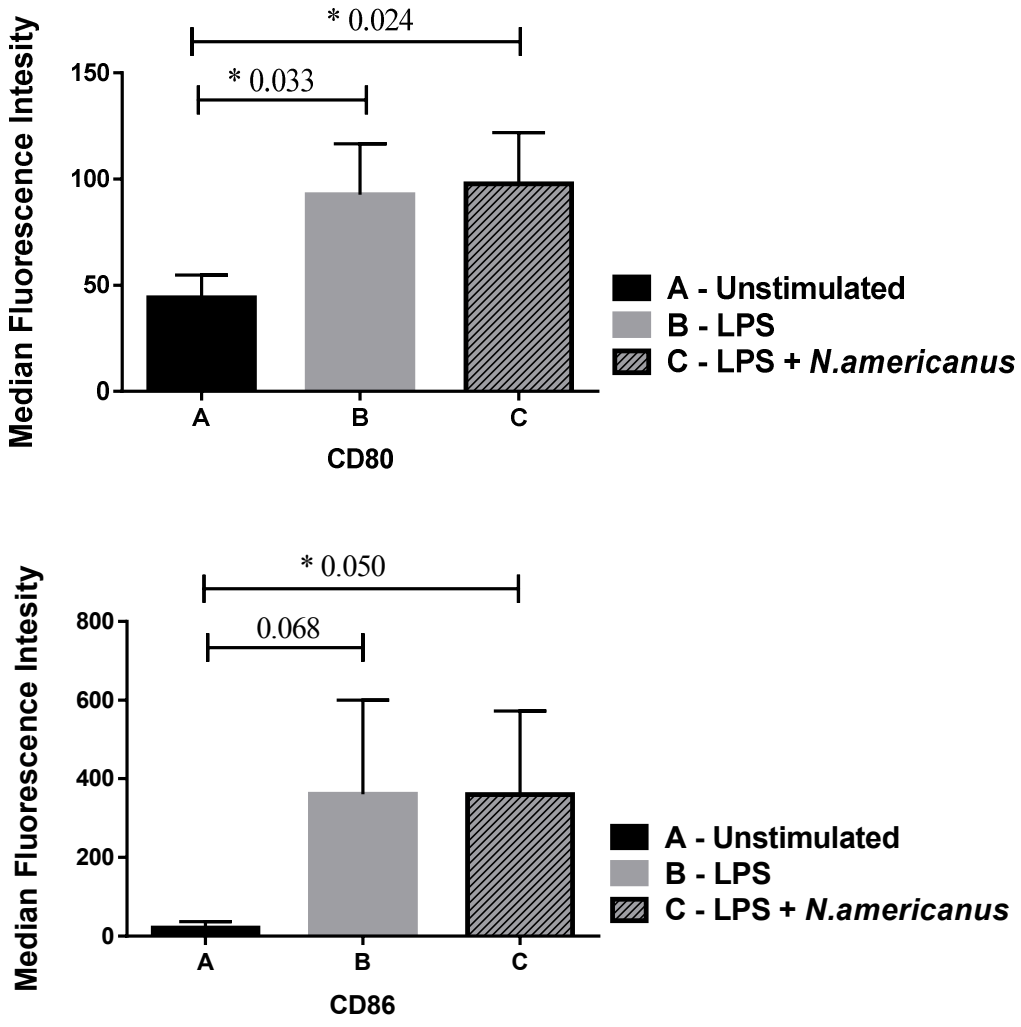


Figure 36: The expression of dendritic cell receptor molecules CD80 and CD86 in response to stimulation with LPS or *Necator americanus* and LPS (± SEM).

DCs exhibited no impairment in responding to LPS in co-stimulatory conditions. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

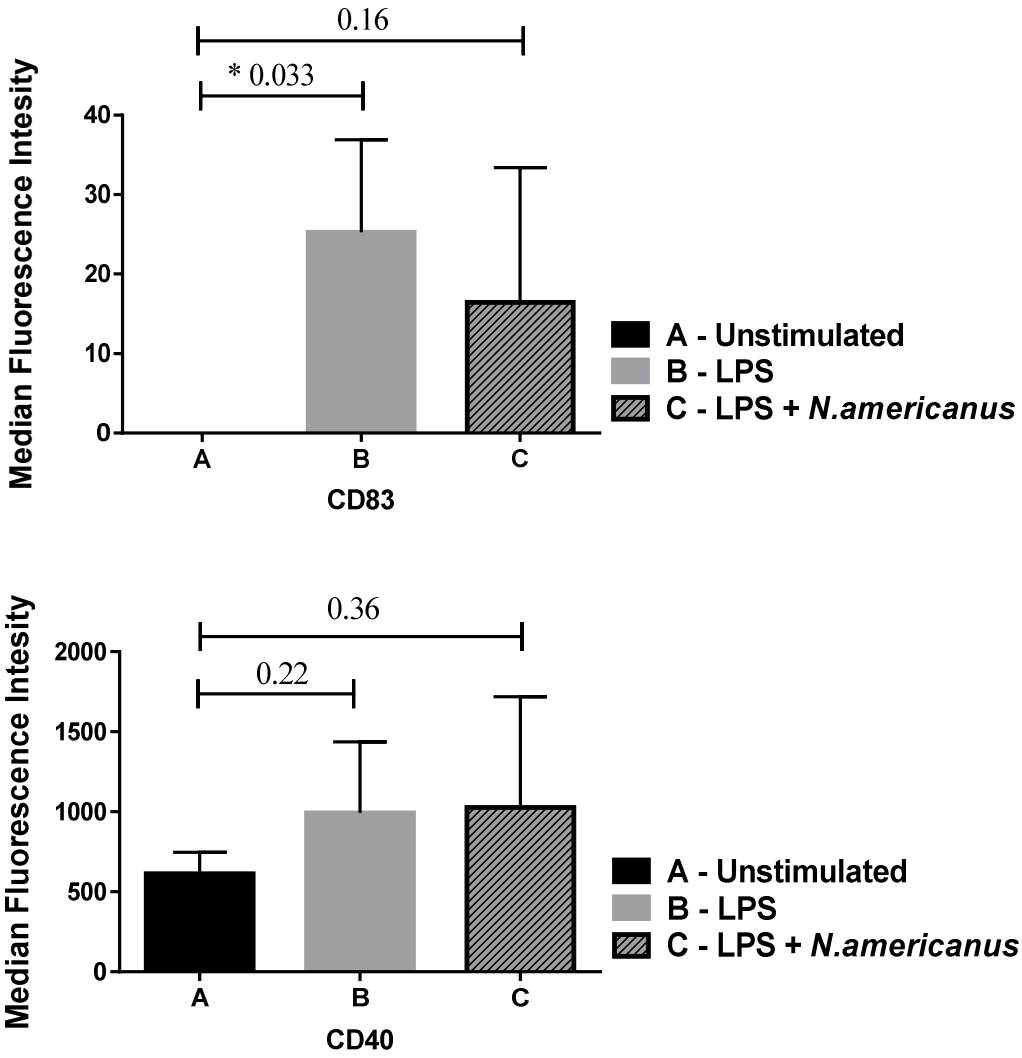


Figure 37: The expression of dendritic cell receptor molecules CD83 and CD40 in response to stimulation with LPS or *Necator americanus* and LPS (± SEM).

DCs exhibited no impairment in responding to LPS in co-stimulatory conditions. However the data was varied, with large ±SEM, due to the small number of donors. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

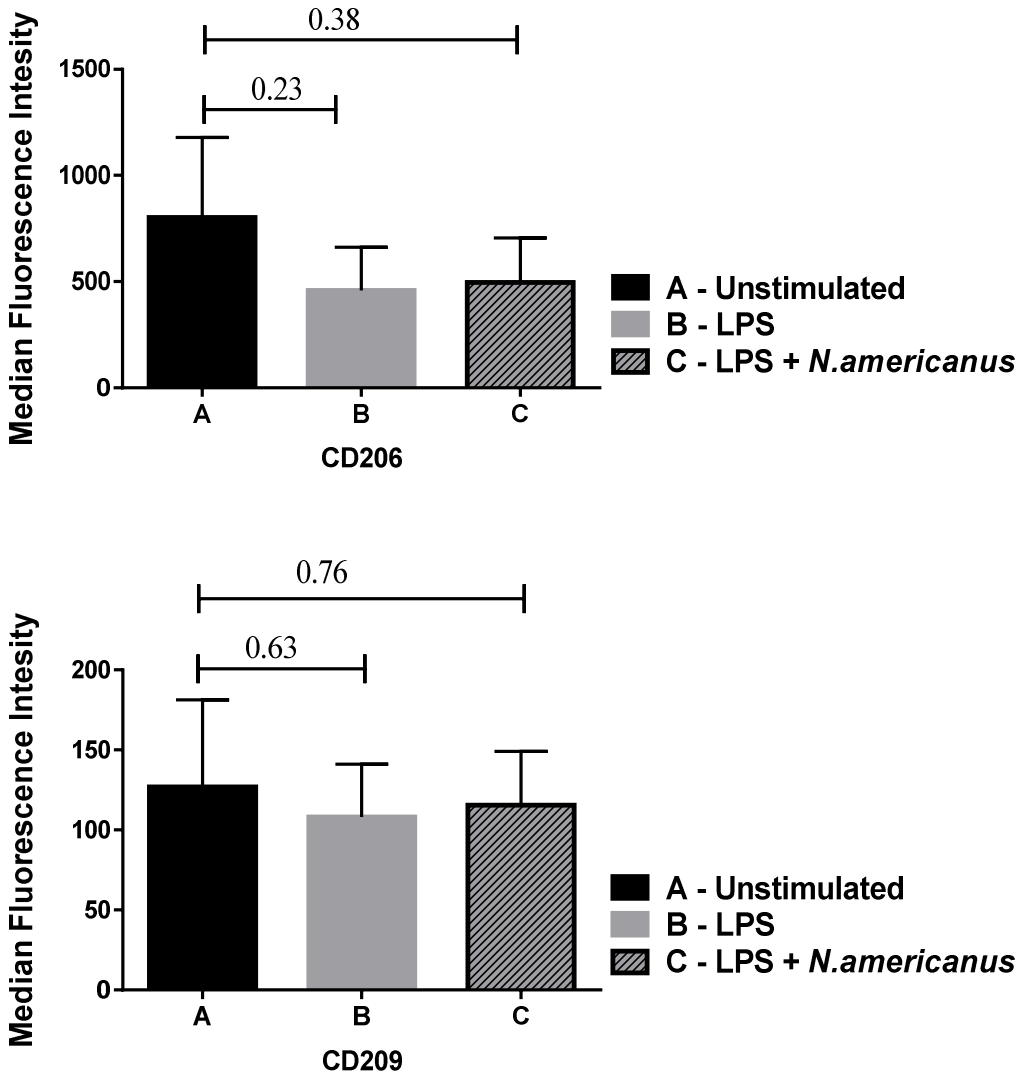


Figure 38: The expression of dendritic cell receptor molecules CD206 and CD209 in response to stimulation with LPS or *Necator americanus* and LPS (± SEM).

DCs exhibited an overall decrease in response to LPS and co-stimulatory conditions. However the data was varied, with large ±SEM, due to the small number of donors. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

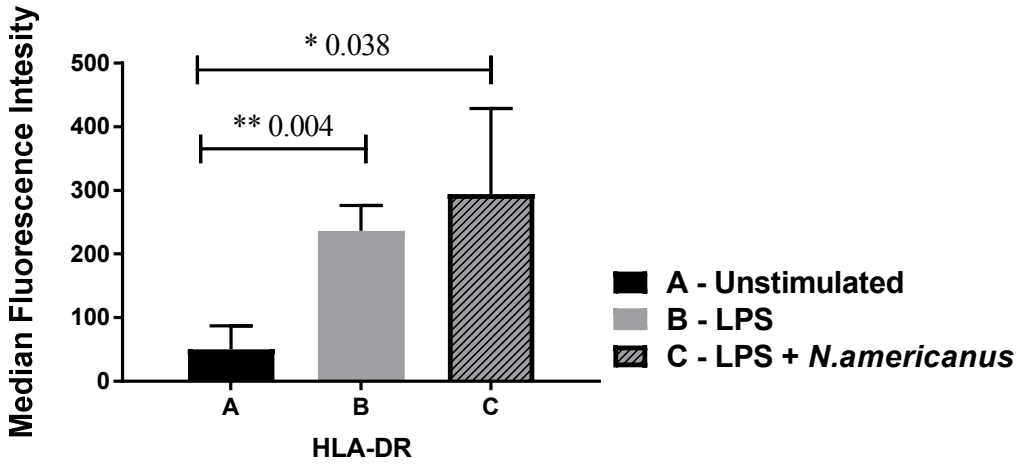


Figure 39: The expression of dendritic cell receptor molecules HLA-DR in response to stimulation with LPS or *Necator americanus* and LPS (± SEM).

DCs exhibited no impairment in responding to LPS in co-stimulatory conditions. Data are representative of 3 independent experiments using DCs from 3 different donors and 3 larvae batches (n=3).

3.1.7 Dendritic cell cytokine expression in response to *Necator americanus* and LPS stimulation

Following the stimulation of DCs with either the infective L_3 *N. americanus* larvae, LPS or both, the supernatant samples were collected at 24 hours and were analysed for IL6, 8, 10 and 12 using ELISA development kits. The data show that while axenic *N. americanus* larvae on its own do not induce any cytokine production by DCs, they suppress LPS induced cytokine expression. However, this suppression did not reach statistical significance (p value ≤ 0.3).

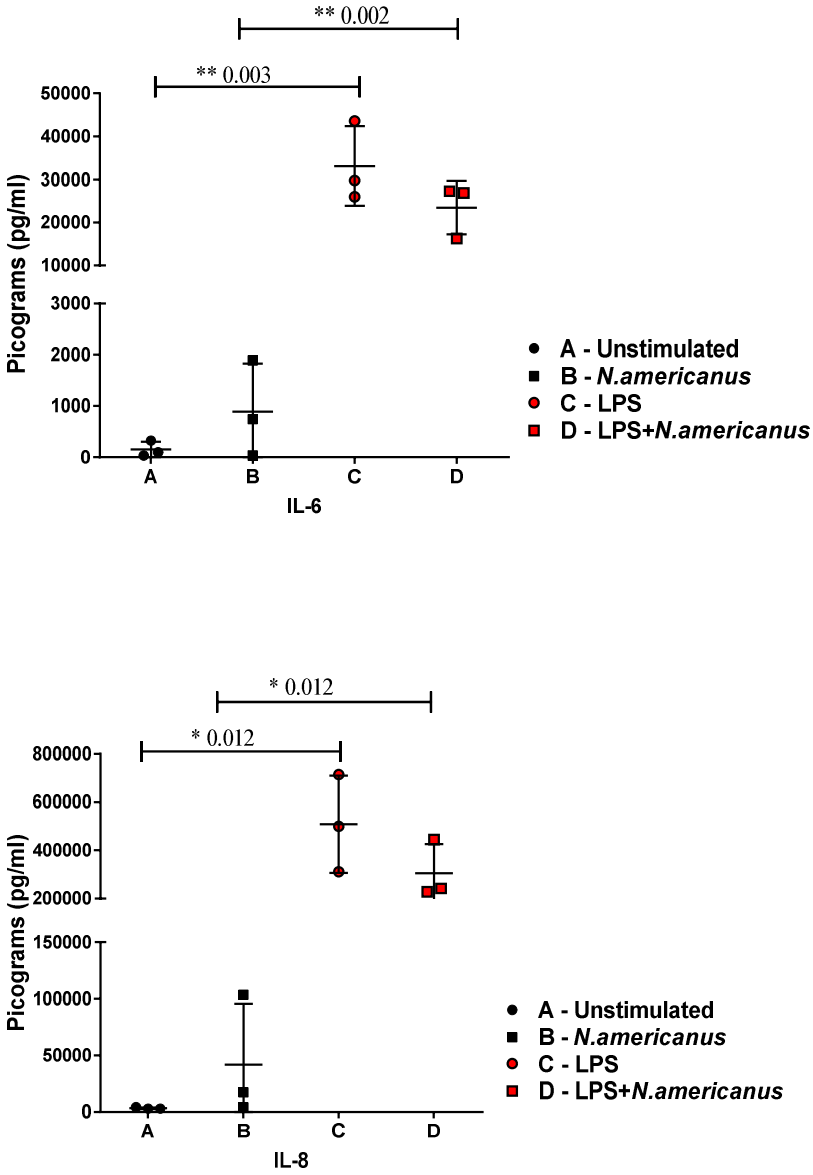


Figure 40: The expression of cytokines IL-6 and IL-8 by dendritic cells in response to stimulation with LPS and *Necator americanus* (± SEM).

DCs stimulated with the infective larvae exhibited very low levels of IL-6 and IL-8 expression. However in co-stimulatory conditions there is a non-significant (p value ≤ 0.3) suppression in LPS induced cytokine expression. Data shown are representative of 3 independent experiments.

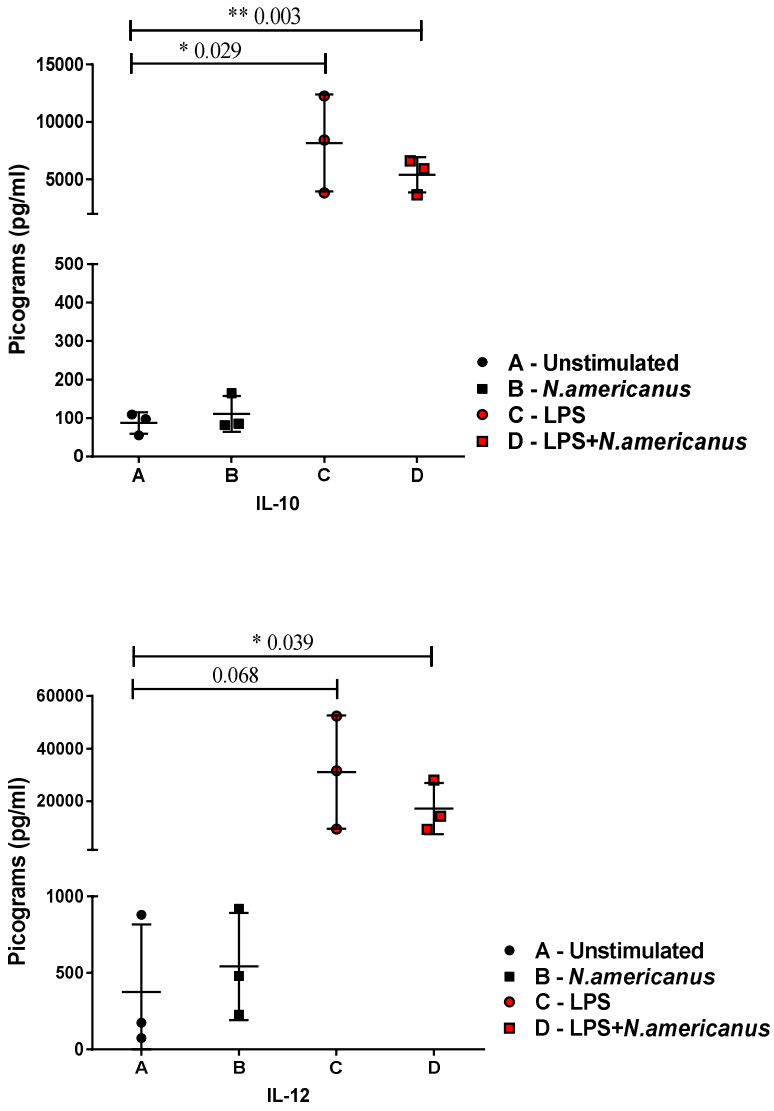


Figure 41: The expression of cytokines IL-10 and IL-12 by dendritic cells in response to stimulation with LPS and *Necator americanus* (± SEM).

DCs stimulated with the *N. americanus* larvae exhibited very low levels of IL-10 and IL-12 expression. However in co-stimulatory conditions there is a non-significant (p value ≤ 0.3) suppression in LPS induced cytokine expression. Data shown are representative of 3 independent experiments.

3.1.8 Cytotoxicity assessment of *Necator americanus* on dendritic cell viability

Cells, when healthy, have an intact cell membrane which doesn't leak any of its intracellular components. However, during the early stages of apoptosis, membrane phosphatidylserine (PS) is translocated from the inner plasma membrane to the cell surface, effectively exposing the PS to the extracellular space (Vermes, Haanen et al. 1995, van Engeland, Nieland et al. 1998). Annexin-V (conjugated to a fluorochrome) has a high binding affinity for PS, which can therefore be employed as a marker for cell membrane integrity (Vermes, Haanen et al. 1995, van Engeland, Nieland et al. 1998). Annexin V is applied in combination with propidium iodide (PI) as later stages of apoptosis are characterized by significant membrane damage where the cells become permeable to PI. Once PI has permeated the cells, it intercalates with DNA, which upon binding, induces PI fluorescence (Riccardi and Nicoletti 2006). This cytotoxicity assay distinguishes between apoptotic cells (Annexin V^+ and PI^-) and non-viable cells (Annexin V^+ and PI^+).

DCs treated with 50 L_3 *N. americanus* larvae for 24 hours were therefore stained for Annexin V FITC and PI (Apoptosis Detection Kit, Beckman Coulter). The data illustrated that DCs remain viable in culture with the *N. americanus* larvae, with a high percentage of cells staining negative for Annexin V and PI (Annexin V^- and PI^-) (Figure 42).

Untreated	*Necator americanus*

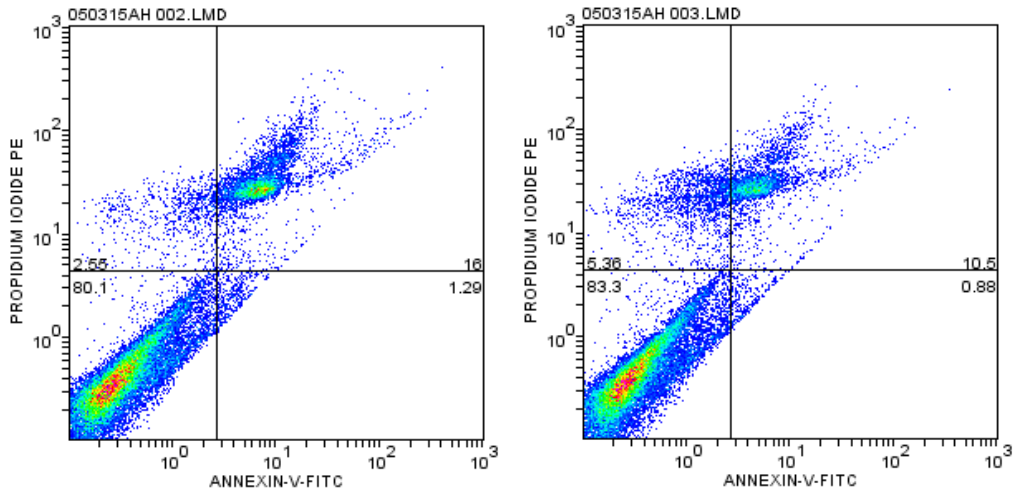


Figure 42: The viability of DCs post treatment with *Necator americanus* larvae.

DCs remained viable post 24 hour treatment with the *N. americanus* larvae staining 80%> negative for Annexin V FITC and PI. Data shown are representative of 3 independent experiments (n=3).

3.2 Discussion

3.2.1 The biological relevance and benefit of the *Necator americanus* larvae sheath during the initial stages of infection

The present thesis provides new insights into early immunological events at the interface of DCs and *N. americanus* larvae and could explain how L_3 evade immunity upon initial interaction with APCs. The exsheathing of *N. americanus* larvae has been observed during the initial stages of infection as well as in the presence of human sweat (Hawdon, Volk et al. 1993, Pasuralertsakul and Ngrenngarmlert 2006), however the factors initiating exsheathing and the benefit of this to the parasite remains to be understood more clearly (Loukas and Prociv 2001). From previous literature, it is known that the sheath does not accompany the hookworm post the skin stages of infection and progression into the blood circulation (Behnke, Paul et al. 1986, Kumar and Pritchard 1992, Hawdon, Volk et al. 1993). This implies that the ensheathed larvae encounter immune cells in the skin *in vivo*. Additionally, early research has identified a range of lectins isolated from plants capable of binding to sugars present on the L_3 sheath, including mannose, fucose, heparin sulphate and galactose (Kumar and Pritchard 1992). Rationally, the biological relevance of the glycosylated larvae sheath and the purposes of exsheathing were investigated.

The data presented in this chapter for the first time show that immature DCs bind to the ensheathed larvae, initiating the *N. americanus* to exsheath and mechanically migrate away from this site, leaving behind its cuticle. DCs are sequestered onto the discarded sheath and form aggregates, exhibiting no preference to binding to the exposed larvae. These findings allude to a disparity between the surface chemistry of the sheath and larvae; a possibility for their differential ability to interact with DCs. Fundamentally, this also provides an explanation to early research which reported that antibodies from infected individuals were only capable of recognising the antigens on the surface of the sheath, but not the exposed larva (Pritchard, Quinnell et al. 1990). While the exact nature of differences in the surface properties of the larvae and sheath are yet to be characterised in detail, the data clearly indicates the presence of distinct chemical signatures on the *N. americanus* cuticle sheath that attract DCs. Consequently this enables larvae migration without being recognised or challenged by APCs.

Furthermore, as part of their armoury of PRRs, DCs express a range of CLRs with a specificity for the recognition of glycosylated antigens (Thompson, Kaminski et al. 2011). Amongst CLRs expressed by DCs are DC-SIGN and mannose receptor (MR or CD206) both of which have been implicated in key DC functions including mediating immune responses to different pathogens as well as immune modulation (Garcia-Vallejo and van Kooyk , Geijtenbeek, Engering et al. 2002, Wollenberg, Mommaas et al. 2002, Salazar, Sewell et al. 2013). Given the high expression of DC-SIGN and MR on myeloid DCs and

their prominent role in recognition of different pathogens, their potential role in DCs interaction with *N. americanus* larvae were investigated using antibodies to block MR and DC-SIGN. The data illustrated that DCs treated with α-DC-SIGN, α-MR or mannan (a ligand for both DC-SIGN and MR (Salazar, Sewell et al. 2013)) exhibit a significant decrease in induction of larvae exsheathing and forming aggregates around cuticles, compared to those observed in the presence of untreated DCs. This clearly indicates a role for MR and DC-SIGN in mediating interactions between DCs and the larvae. Intriguingly, the blocking of either of these receptors results in the prevention of exsheathing and DCs aggregate formation around the larvae sheath. This establishes a mandatory interaction of both DC-SIGN and MR on DCs, with the glycosylated larvae sheath.

Notably in untreated conditions, the initial DCs which are sequestered onto the sheath form dense aggregates, where bystander cells part of the aggregate are not in direct contact with the larvae sheath. This could be due to the role of DC-SIGN and MR in adhesion. Primarily, DC-SIGN functions as an adhesion molecule with a high affinity for ICAM-3 to support DC interactions with T cells, and ICAM-2 promoting trans-endothelial migration (Geijtenbeek, Krooshoop et al. 2000). Additionally, *Borrelia burgdorferi* has been shown to employ the MR to adhere firmly to monocyte derived macrophages and MR-transfected rat cells (Cinco, Cini et al. 2001). Albeit the dense cell aggregates formed could be due to a CLR mediated adhesion cascade, the exact mechanism requires further clarifications.

Furthermore, the inhibition of DC sequestration onto the larvae sheath (after blocking with α-CLRs or mannan), is reversed after prolonged periods. Upon ligand or antibody binding, DC-SIGN and MR are rapidly internalized, following which these CLRs are recycled consecutively to the cell surface and mediate further ligand internalization (Stahl, Schlesinger et al. 1980, Engering, Geijtenbeek et al. 2002, Tacken, Ter Huurne et al. 2012). It can therefore be inferred that due to receptor recycling of DC-SIGN and MR, these CLRs are possibly available (after 48 hours) to interact with and bind to the glycosylated *N. americanus* sheath.

An escape mechanism through which the larvae could possibly bypass the immune cells is depicted in Figure 43. The proposed mechanism provides a plausible explanation for the poor T cell responses observed *in vivo*. The sequestration of DCs onto larvae sheaths, via interactions with DC-SIGN and MR, would hypothetically prevent necessary migration to lymph nodes. This would obstruct the effective stimulation of the adaptive immune responses (Martin-Fontecha, Lanzavecchia et al. 2009).

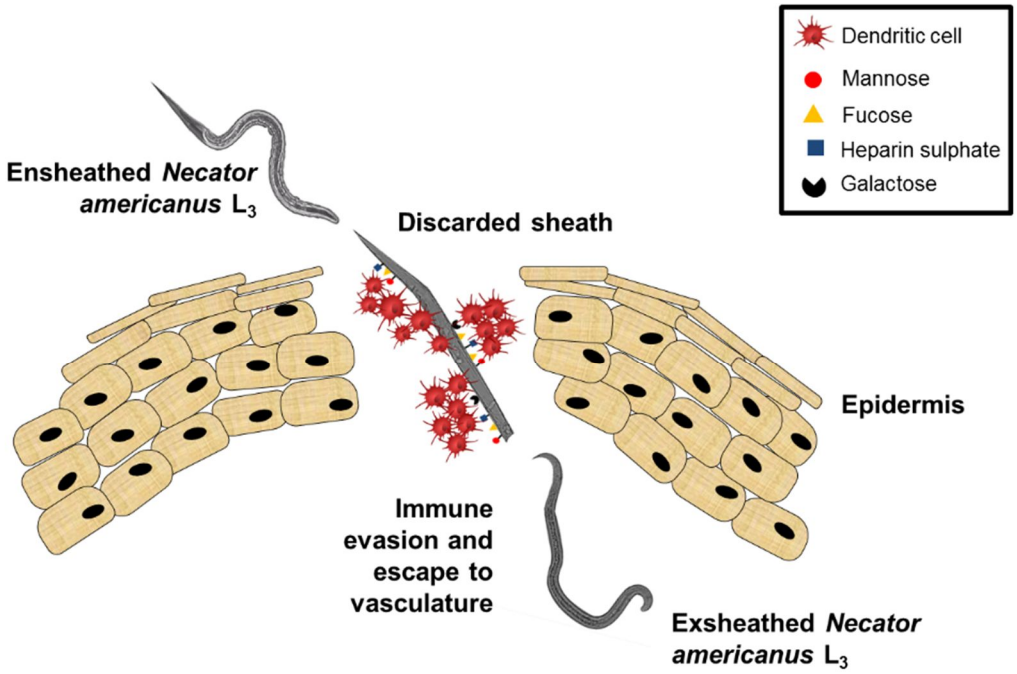


Figure 43: The proposed mechanism of *Necator americanus* immune evasion strategy.

DCs bind the *N. americanus* sheath via CLRs which triggers the exsheathing of the larvae. The DCs are sequestered onto the discarded sheath and the larvae escapes to the vasculature.

DC-SIGN and MR have been implicated in key DC functions including mediating immune responses to different pathogens as well as immune modulation (Garcia-Vallejo and van Kooyk , Geijtenbeek, Engering et al. 2002, Wollenberg, Mommaas et al. 2002, Salazar, Sewell et al. 2013). Previous studies have shown that some pathogens subvert DC-SIGN function in order to evade immune detection and surveillance. These include viral pathogens, such as HIV-1 via gp120 protein, and non-viral pathogens including *Mycobacterium tuberculosis* (van Kooyk and Geijtenbeek 2003, Ludwig, Lekkerkerker et al. 2004). In the context of Necatoriasis, the infective larvae could possibly be exploiting the MR or the unique function of DC-SIGN. The latter can initiate

distinct signalling pathways and immune responses, through the recognition of different carbohydrate groups (i.e., mannose or fucose), expressed by the parasitic (Gringhuis, Kaptein et al. 2014). For instance, a shared feature among many patristic helminths is their ability to inhibit Th1 responses, and polarise the immune response towards a Th2 phenotype (Loukas and Prociv 2001, Geiger, Caldas et al. 2007, McSorley and Maizels 2012, McSorley, Hewitson et al. 2013). Previous studies in helminths have reported MR internalization of Omega-1, a *Schistosoma mansoni* derived, glycosylated ribonuclease. The glycoprotein is internalized and functions to degrade ribosomal and messenger RNA, which interferes with DCs protein synthesis, conditioning the DCs for a Th2 phenotype (Everts, Hussaarts et al. 2012). Other research have also shown *Schistosoma mansoni* helminths to polarise DC-SIGN immune responses towards a Th2 phenotype through recognition of glycan antigen Lewis x (van Die, van Vliet et al. 2003). This initiates a TLR4 induced immune response through the regulation of BCL-3, which is translocated to the cell nucleus. The translocation of BCL-3 promotes a Th2 polarization and enhances chemokine expression of CCL17, 22 and anti-inflammatory IL-10 with a suppression in pro-inflammatory cytokines produced (Gringhuis, Kaptein et al. 2014).

These glycosylated residues (i.e., mannose and fucose) are present on the larvae sheath (Kumar and Pritchard 1992), and the data clearly suggests that the *N. americanus* are actively targeting DC-SIGN and MR to sequester DCs onto the larvae sheath. Nonetheless, the exact nature of these interactions remain to be established.

3.2.2 The intracellular lysis of *Necator americanus* by mature dendritic cells

DCs were matured in the presence of a potent stimulant, LPS, which ensures the secretion of inflammatory cytokines and chemokines in the culture media. These include including IL-6, 8, 12 and TNF-α (Abdi, Singh et al. 2012). The treatment of *N. americanus* larvae with LPS-conditioned (cell-free) media from mature DCs had no effects on larvae exsheathing and viability. Additionally, culture media from DCs treated with mannan (to stimulate CLR ligation) were also ineffective in inducing larval exsheathing, which collectively indicated a necessary cell mediated interaction. For the first time, the data presented in this chapter has illustrated that, albeit LPS-conditioned media had no effect on the larvae; mature DCs have the capability to lyse and render infective L_3 *N. americanus* non-viable. It is imperative to note that this culture media did not contain LPS and that the mature DCs were not physically interacting with the larvae.

Primarily, the maturation process of DCs is complex with various phenotypical modifications. These include the loss of phagocytic/endocytic receptors, the upregulation of maturation markers and the secretion of chemokines and cytokines. Modifications which function together to ensure differentiation and polarization of the target immune cells (Ueno, Klechevsky et al. 2007). The expression of both DC-SIGN and MR, are regarded as a differentiation hallmark receport for immature DCs, which are downregulated following antigen

encounter and maturation (Wollenberg, Mommaas et al. 2002, Garcia-Vallejo and van Kooyk 2013). In this context, following LPS treatment, the level of DC-SIGN and MR available to interact with the larvae sheath would have decreased significantly (Wollenberg, Mommaas et al. 2002, Garcia-Vallejo and van Kooyk 2013). Rationally, the lack of cell aggregates observed in culture with mature DCs and *N. americanus* larvae is potentially due to low expression of these CLRs.

However, in the instance where DCs encounters both cytokines and pathogens, previous studies have shown that the paracrine signalling response is modified. For instance, DCs infected with the Newcastle's Disease Virus (NDV) secrete cytokines which stimulate naïve DCs to become partially activated and moderately resistant to the virus (Bordería, Hartmann et al. 2008, Hartmann, Marjanovic et al. 2014). A process whereby the immune cells are generating an enhanced rapid response to the NDV. The study also reported that a specific combination of cytokines secreted can modify DC responses; a mechanism which proposes that the cytokine microenvironment acts directly on the immune cells to navigate the immune responses generated (Hartmann, Marjanovic et al. 2014). It is therefore within reason to assume that the cytokine secretions by mature DCs in response to LPS differ to those in culture with *N. americanus*, as the latter renders the larvae non-viable. A plausible mechanism by which the mature immune cells adapt and modify their secretions via a paracrine signalling pathway, to directly eliminate and lyse the *N. americanus* larvae. Nevertheless, the DCs in the peripheral tissues such as skin, are immature, with developmental

changes initiated following pathogen encounter and subsequent DC maturation (van Vliet, den Dunnen et al. 2007).

3.2.3 The effect of *Necator americanus* larvae on dendritic cell phenotype

A pivotal observation following the treatment of immature DCs with *N. americanus* larvae was the significant reduction in MR expression. In contrast all the other tested surface markers (CD80, CD83, CD86, CD40, and HLA-DR) exhibited no change and DCs maintained an immature phenotype. On the other hand, DCs co-cultured with *N. americanus* larvae maintained their ability to respond to LPS stimulation as evidenced by upregulation in maturation markers. This was perhaps a reflection of the axenised state of the larvae that are used in these experiments, which is unlikely to be the case *in vivo (Loukas and Prociv 2001)*. Arguably the findings presented are independent of the larvae axenised state, as there is a specific downregulation in CD206 expression, and the data concur with and expand on previous literature. These studies reported that monocyte derived DCs from infected volunteers (as opposed to non-infected) had a down regulated expression of DC markers i.e., CD86, CD11c, following stimulation with LPS, alluding to parasite impaired DCs in human hosts (Fujiwara, Cancado et al. 2009).

Furthermore, in regards to the lack of maturation observed in response to the larvae, it is imperative to note that the maturation status of DCs play a key role

in their ability to initiate T cell activation (Kruse, Meinl et al. 2001, Elgueta, Benson et al. 2009). CD83 is one the best known maturation markers, although its function is still unclear. Nevertheless, studies have shown that a down regulation of CD83 on DCs via RNA interference (Aerts-Toegaert, Heirman et al. 2007) results in a decrease in T cell proliferation, capacity for IFN-γ secretion and priming of antigen specific CD8+ T lymphocytes. Intriguingly, these are all hallmark characteristics of infected individuals with *N. americanus* (Bentwich, Weisman et al. 1996, Kalinkovich, Weisman et al. 1998, Borkow, Leng et al. 2000, Loukas and Prociv 2001). *In vivo*, the lack of upregulation in DC co-stimulatory molecules such as CD80/86 prevents the effective activation of T cells, as these bind TCR and CD28 (Vasilevko, Ghochikyan et al. 2002). Other co-stimulatory molecules found on DCs such as CD40 are necessary for DC activation and priming of Th1 cell differentiations through induction of IL12 production by DCs (Elgueta, Benson et al. 2009). Furthermore, a lack of HLA-DR upregulation in response to foreign antigens prevents further activation of Th cells and the production of antibodies against this specific parasite.

Nevertheless, the specific down-regulation of MR expression on DCs in co-culture with *N. americanus* is of great interest and further highlights the presence of MR ligands on larvae surface. Upon pathogen recognition, MR is internalized together with the bound PAMP derived from the larvae and mediates phagocytosis via the phagocytic pathway (Wollenberg, Mommaas et al. 2002). This could possibly account for the decrease in CD206 expression on DC surface, which is in line with other helminth studies such as *Schistosoma*

mansoni infections. These studies revealed a novel pathway involving the internalization, (decreasing MR expression), of helminth derived glycoproteins through the MR on DCs (Everts, Hussaarts et al. 2012).

3.2.4 The effect of *Necator americanus* larvae on dendritic cell cytokine expression

To further investigate whether the larvae are able to modulate DC responses to other stimuli, the cytokine profile after co-stimulation with LPS, which is likely to be present during any *in vivo* exposure, was studied. DCs are susceptible to cytokine activation, thus their role is crucial. These initiate maturation processes resulting in the migration of DCs to the lymphoid organs where T cells are primed with MHC and peptide complex antigens derived from foreign proteins (Blanco, Palucka et al. 2008). Previous immunoepidemiological studies have investigated a wide range of pro/anti-inflammatory cytokine/chemokine (i.e., IFN-γ, TNF-α, IL-1β, IL-4, IL-6, and IL-10) secretions by DCs in individuals with Necatoriasis. These studies reported no statistical difference in cytokine/chemokine expression by DCs from healthy individuals in comparison to infected volunteers. However whether the larvae were capable of supressing DC cytokine expression in the presence of potent stimulants, was not investigated (Fujiwara, Cancado et al. 2009).

Therefore to expand on this, 4 cytokines were selected; firstly the dual functioning IL-6, which has been shown to increase and decrease inflammation. This cytokine is crucial for autoimmunity and inflammation, having significant roles in Th17 differentiation (Scheller, Chalaris et al. 2011, Mihara, Hashizume et al. 2012). The chemokine or neutrophil chemotactic factor IL-8, primarily stimulating chemotaxis, where there is an increase in vascular permeability and the predominant migration of neutrophils to the site of infection (Waugh and Wilson 2008). The anti-inflammatory cytokine IL-10, as it has been shown to have major roles in downregulating and inhibiting Th1 cytokines, including IL-2 and 3, GM-CSF, IFN-γ and TNF-α (Couper, Blount et al. 2008). Finally IL-12 due to its function in regulating Th1 and 2 responses, effectively stimulating Th1 cells to secrete IFN- γ (Miro, Nobile et al. 2006, Kaka, Foster et al. 2008).

The data from these experiments indicated a general suppression in DC responses to LPS in the presence of larvae. These included IL-6, 8, 10 and 12, albeit not reaching a statistical significance (p value ≤0.3). However this was not due to any changes in DC viability as these stained Annexin V^- and PI^- negative. Interestingly, early research conducted in *N. americanus* endemic areas of Brazil, found that infected individuals secreted lower levels of cytokines (IL-5, IL-10, IL-13 and CXCL10), comparable to healthy volunteers (Geiger, Caldas et al. 2007). This study concluded that the larvae are capable of stage-specific down regulation of immune responses within the host. Rationally, the suppression in LPS induced cytokine expression by DCs is more than likely due to this immune suppressive factor associated with the larvae.

Considering the literature available on human hookworm infection, there is a recurrent theme surrounding *N. americanus* immunomodulatory properties i.e., (Loukas and Maizels 2000, Geiger, Caldas et al. 2007, Fujiwara, Cancado et al. 2009, Reddy and Fried 2009) etc. Collectively, the findings presented within this chapter are in agreement, and identify DC-SIGN and MR as key receptors targeted by the larvae to modulate innate immune responses within the host.

3.2.5 Conclusion and future work

The data presented in this chapter provides novel insights into *N. americanus* interactions with pivotal cells of the immune system, identifying exciting areas for further research.

Primarily, the novel finding of larvae lysing capability by mature DCs, highlights this as an imperative area for future work. The identification of specific combinatory cytokine/chemokine secretions in the microenvironment by mature DCs (in culture with the larvae), could possibly pave the way for more efficient intervention strategies against hookworm infection. To date the only human hookworm vaccine with promising outcomes, is being developed by the Sabin institute. However this bivalent vaccine, currently in stage 1 clinical trials, targets the chronically impairing symptoms associated with the later stages of infection (Sabin PDP 2017). The identification of which specific cytokines mature DCs secrete to modify immune responses, in order to lyse the larvae,

could potentially be utilised in vaccines targeting the initial stages of infection. Additionally, investigating the effects of mature DCs culture media (following incubation with the larvae) on live ensheathed *N. americanus* hookworms is vital. This will elucidate whether the novel lysing of the hookworms is due to a secreted factor or co-dependent on the presence of mature DCs.

The data discussed in this chapter also provides strong rationale for further experiments targeting specific lectins on larvae surface, and/or silencing MR and DC-SIGN expression on DCs. This will fully elucidate the cross-talk between the sugar moieties on larvae sheath and the CLRs on DCs. In parallel a microarray analysis of known DC ligands on the larvae sheath, would highlight possible receptor-ligand interactions. It is also fundamental to assess *N. americanus* treated DCs capability to effectively prime naïve T cells in co-culture studies. The sequestration of DCs onto the larvae sheath would in theory prevent DCs interacting with T cells. In addition, the lack of co-stimulatory receptor molecules following larvae treatment would hamper DCs capability to stimulate T cell responses *in vitro*. This will provide further insight into the poor adaptive immune responses observed *in vivo*, as the exact mechanism for larvae induced downregulation of T cell reactivity are under researched (Geiger, Caldas et al. 2007).

Another key area for research is the interaction of *N. americanus* larvae with other components of the immune system. This could identify whether the larvae sheath is adapted and tailored for bypassing other immune cells such as

monocytes, macrophages, eosinophils, Langerhans and mast cells (Chaplin 2010). Eosinophilia, in particular is a hallmark characteristic of *N. americanus* infection (Wright and Bickle 2005), which is thought to bind to the L_3 larvae, due to the predominant and significant increase of these inflammatory mediators at the site of infection.

Finally, the larvae employed in this thesis for research were ensheathed and axenised, the latter of which is not the case *in vivo* (Loukas and Prociv 2001). Therefore, to provide a better immunological understanding between the larvae and components of the innate immune system, it would be highly recommended to employ un-sterile hookworms for further research. In addition, co-culturing exsheathed *N. americanus* with immature DCs, could identify whether these APCs cells are capable of interacting with the exposed larva in the absence of the cuticle sheath.

Monocyte derived dendritic cells phenotype and function							
DC response to *Necator americanus*	CD80 No upregulation	CD86 No upregulation	CD83 No upregulation	CD40 No upregulation	HLA-DR No upregulation	CD206 Significantly downregulated P value *0.052	CD209 No difference
DC response to LPS and co-stimulation with *N. americanus* + LPS	CD80 Upregulation P values *0.029 and 0.090	CD83 Upregulation P values *0.031 and 0.075	CD40 over expressed P values *0.019 and **0.002	CD86 over expressed P values 0.073 and **0.001	HLA-DR Upregulation P values **0.003 and *0.014	CD206 Down regulation P values 0.1 and *0.035	CD209 Overall downregulation
Cytokine expression by DC in response to *N. americanus* Vs LPS	IL-6 P value **0.003	IL-8 P value *0.012	Il-10 P value *0.029	Il-10 P value 0.068	DC simultaneously treated with LPS and *N. americanus* exhibited overall non-significant suppression in cytokine expression in comparison to LPS stimulated DC. Mature DCs lyse *N. americanus*		
C Type Lectin blocking 24 hours							
Untreated DC	**α-MR**	**α-DC-SIGN**	**Mannan**				
Aggregate	No aggregate	No aggregate	No aggregate				

Table 1: Summary of dendritic cells and *Necator americanus* interactions.

CHAPTER 4: *Necator americanus* and epidermal keratinocytes

4.1 Results

In this chapter, the primary objective of the work conducted was to elucidate the physical interactions between infective L_3 *N. americanus* larvae and epidermal keratinocytes (HaCat). However, due to the inconsistent and limited source of larvae availability (Tanasescu and Constantinescu 2015), several fundamental observations require further research. These are addressed in the discussion, with recommendations made for future work.

The data in Chapter 3 has identified the fundamental role of larvae sheaths, in sequestrating potent DCs. Nevertheless, prior to encountering immune cells (*in vivo*), *N. americanus* larvae burrow into the anatomical barrier, namely the skin. The exsheathment of *N. americanus* has been observed during the initial stages of infection as well as in the presence of human sweat (Kumar and Pritchard 1992, Hawdon, Volk et al. 1993, Pasuralertsakul and Ngrenngarmlert 2006). Although early research considered that larvae are utilising their sheath as an antigenic diversion i.e., DCs sequestration, the factors initiating exsheathment, pre/post-skin penetration remain to be understood more clearly (Loukas and Prociv 2001). Chapter 4 will therefore examine interactions between *N. americanus* larvae and epidermal keratinocytes (HaCat), assessing larval behaviour, skin penetration and exsheathing.

4.1.1 The interaction between *Necator americanus* and human epidermal keratinocytes (HaCat)

In the first instance, the behaviour of *N. americanus* in the presence of epidermal keratinocytes, were assessed by depositing 50 ensheathed larvae onto a confluent layer of HaCat cells. These were cultured at air-liquid-interface on a 12 mm transwell plate with a polycarbonate membrane inserts (0.4 μm pore size). In addition to simulating the barrier function of the epidermis, such experimental set up also ensured larvae were incapable of burrowing into the monolayer of cells (i.e., through the polycarbonate membrane). The larvae are approximately 300-400 μm in size, and cannot penetrate the membrane by means of burrowing. This provides an ideal platform for observing larvae behaviour and exsheathing quantification.

The primary observation was larval dormancy, as the *N. americanus* exhibited less dynamic movements and were exsheathing lethargically in culture with HaCat cells (Figures 44 and 45). At 2 hours post incubation, the larvae remained ensheathed, with minimal movement observed. At 24 hours, only 18 ±3, out of a total of 50 deposited larvae had exsheathed (Figure 44). Consequently, the speed of total larvae exsheathing was then compared in DC and HaCat cell cultures, where a total of 50 ensheathed *N. americanus* larvae were incubated with each cell type. Notably, the larvae were exsheathing at a significantly lower rate in culture with HaCat cells (p value ≤ 0.01), with some larvae remaining

ensheathed, in a resting posture with limited motility, for up to 96 hours (Figure 45).

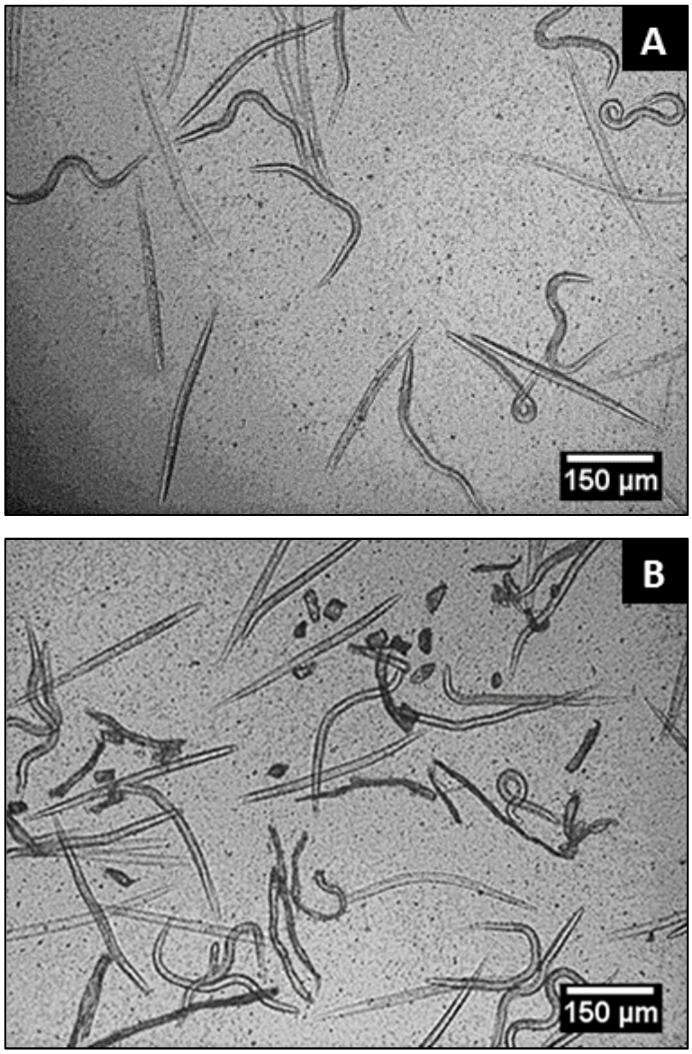


Figure 44: The exsheathing of *Necator americanus* on HaCat cells at 2 hour (A) and 24 hours (B).

Hookworm larvae remain dormant and ensheathed 2 hours post incubation with HaCat cells (A). At 24 hours, approximately 18 ± 3 out of a total of 50 deposited larvae were exsheathed. However the larvae are mainly dormant, exsheathing lethargically and at very slow rates. Data shown are representative of 3 independent experiments and 3 larvae batches.

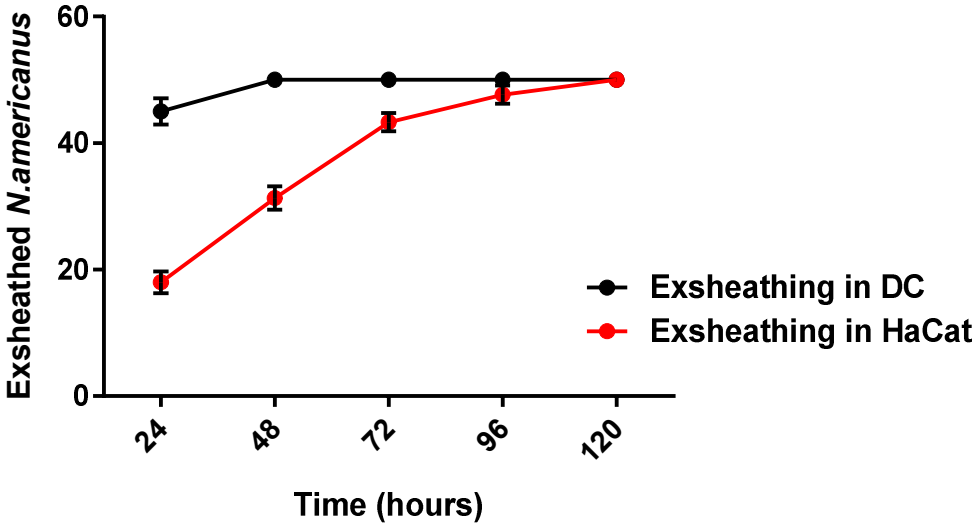


Figure 45: The quantification of *Necator americanus* exsheathing.

A total of 50 *N. americanus* larvae were incubated in culture with DCs and HaCat cells. All hookworms were exsheathed at 48 hours in DC cultures, contrasting HaCat cell cultures, where some larvae remained ensheathed for up to 96 hours. Overall, *N. americanus* larvae in HaCat cell cultures were exsheathing at a significantly lower rate, exhibiting larval dormancy (p value $\leq$ 0.01). Data shown are representative of 3 independent experiments and 3 larvae batches.

Additionally, as with DCs, the exsheathing of an individual *N. americanus* larva was documented in a series of snap-shot images highlighting each stage of exsheathing (Figure 46). Albeit this larva was captured exsheathing on the apical surface of the HaCat cells, it is paramount to highlight that the majority of larvae remained ensheathed whilst burrowing into the HaCat tissue layer (Figure 47).

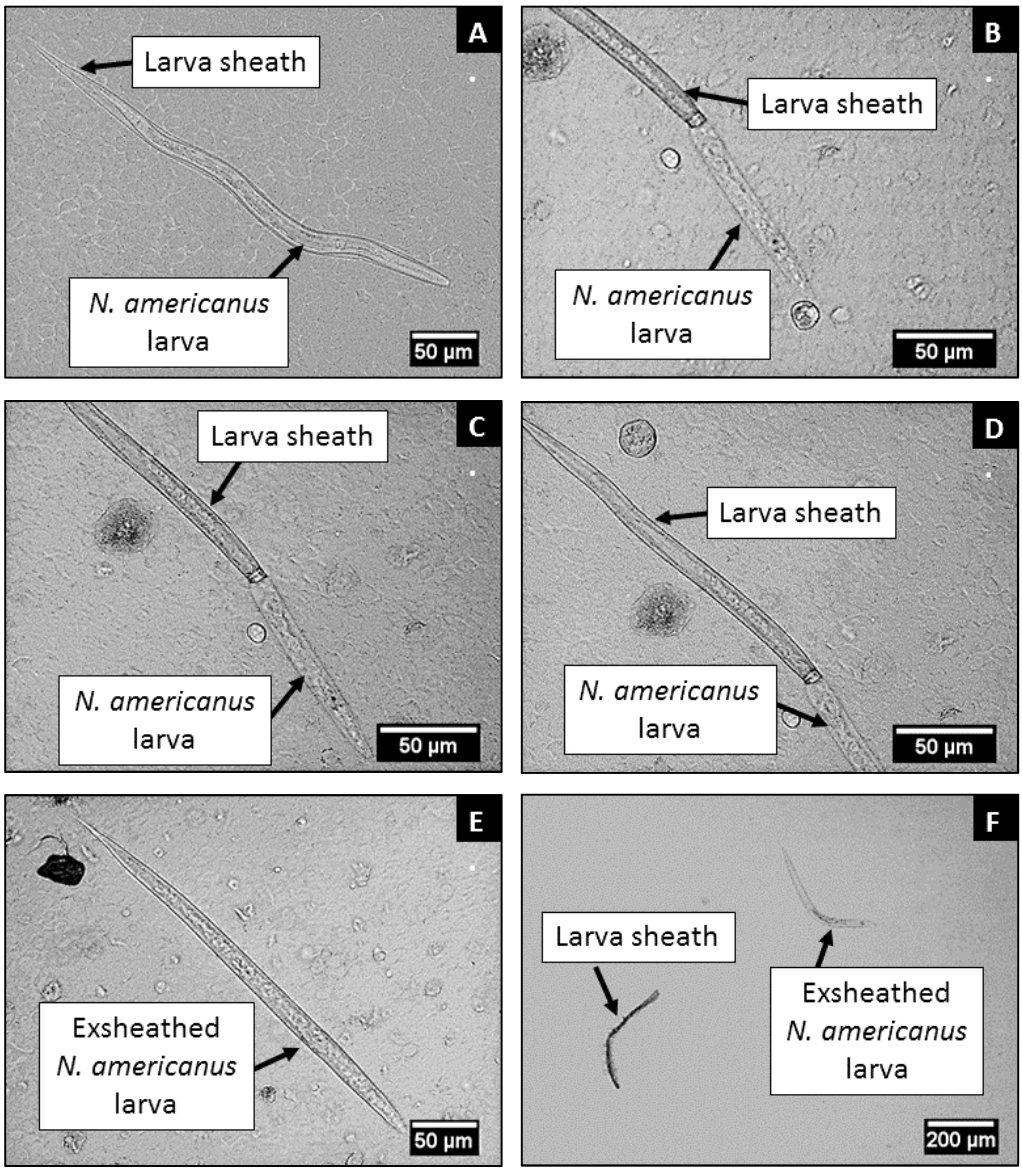
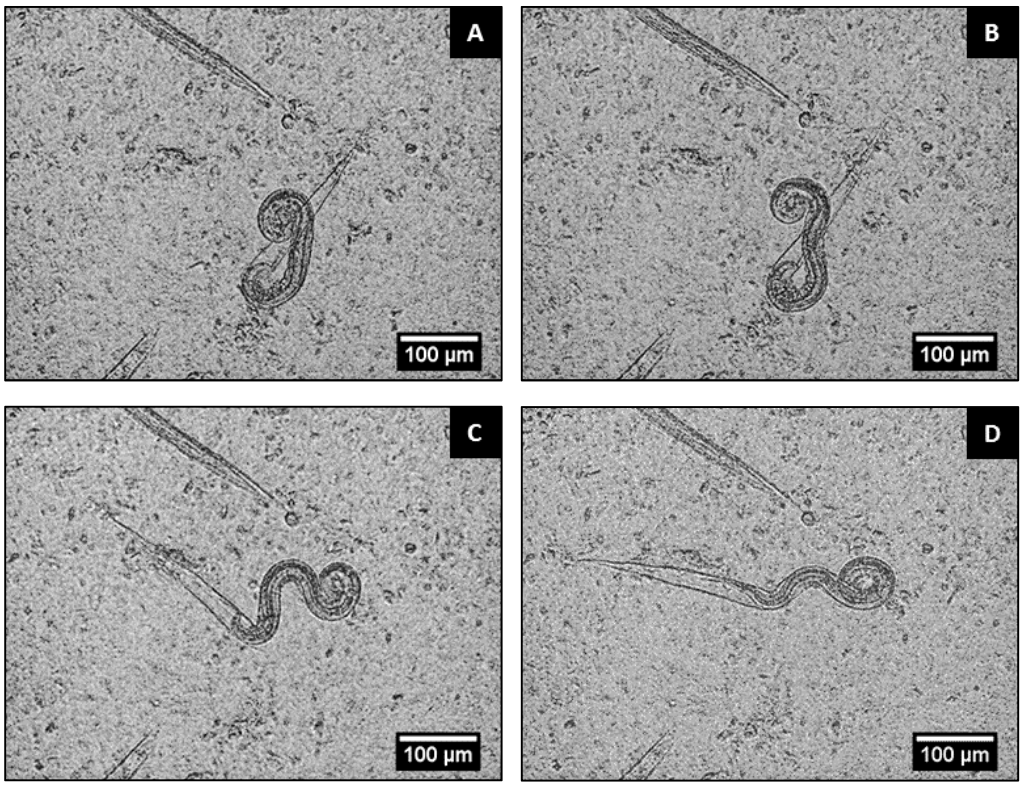


Figure 46: The exsheathing of *Necator americanus* on HaCat cells.

In the presence of HaCat cells, the ensheathed larva remains dormant for a period of 2-4 hours, which can range up to 96 hours (A). Following this period of inactivity, the larva begins to move and the tip of the sheath is broken where the larva emerges from. For this individual larva exsheathing was initiated at 2 hours post incubation (B). The larva alternates between periods of movement and inactivity for 2 minutes, gradually exsheathing (C and D). Once exsheathed at the 3 minutes time point (E), the larva progressively migrates away leaving the discarded sheath behind (D). Data shown are representative of 3 independent experiments and 3 larvae batches.

As shown in Chapter 3 (Figures 19/20 and Video 1), *N. americanus* exsheathing (in culture with DCs), is an instantaneous process following the initial breach of the larvae sheath. Contrastingly, in addition to the overall lower exsheathing rate in comparison to DC cultures, the individual larva incubated with HaCat cells were exsheathing lethargically. Th actual process of exsheathing ranged from 2-5 minutes, with some exiting their cuticle partially, before resuming a dormant state. For such larvae partial exsheathing could last approximately 3 minutes, before resuming inactivity for a further 24-72 hours, while still ensheathed. Data presented in Figure 47 depicts an example of such behaviour.



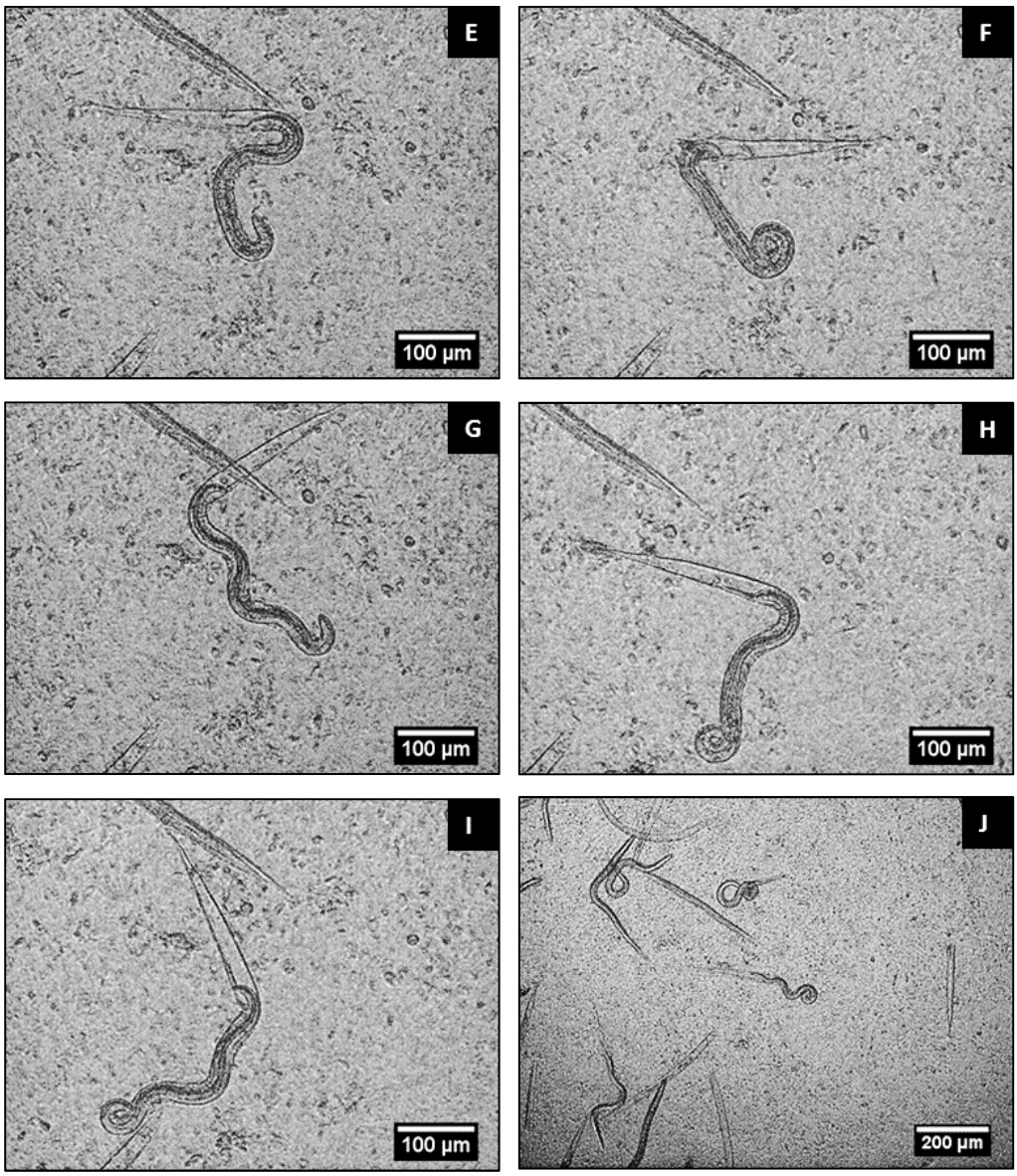


Figure 47: The modified exsheathing behaviour of *Necator americanus* on HaCat cells.

Following the breach of the sheath and initiation of exsheathing, the larva alternates between periods of inactivity and movement. The panels illustrate exsheathing activity of the larva documented within 60, 120 and 180 second intervals (A-C, D-F, and G-I respectively). Panel J illustrates the larva gradually halting exsheathing and re-entering a dormant state. Other *N. americanus* larvae can be observed in this panel which are ensheathed and in a resting posture. Data shown are representative of 3 independent experiments and 3 larvae batches.

Furthermore, the objective of the following experiments was to assess the capability of *N. americanus* larvae to burrow into the HaCat cell layer. In order to evaluate this, the 12 mm transwell plates were substituted with a standard 12 well tissue culture treated plate. This provides a platform which permits larval burrowing and is ideal for observing the mechanical penetration into the keratinocyte tissue layer.

HaCat cells were grown to confluency, following which the cells were incubated with approximately 50 ensheathed *N. americanus* larvae. The data presented in Figure 48, illustrates that albeit some larvae exsheathed (on the apical cell surface) prior to burrowing into the HaCat cell layer, exsheathing was not a mandatory pre-requisite to enable this. Interestingly, some larvae initiated burrowing into the tissue layer, while partially ensheathed. Nevertheless, the majority of the *N. americanus* larvae maintained their sheath, with only a small number of cuticle fragments identified on the apical surface. This alludes to a larval preference, of retaining cuticle sheaths during the initial contact stages with cells of the innate anatomical barrier.

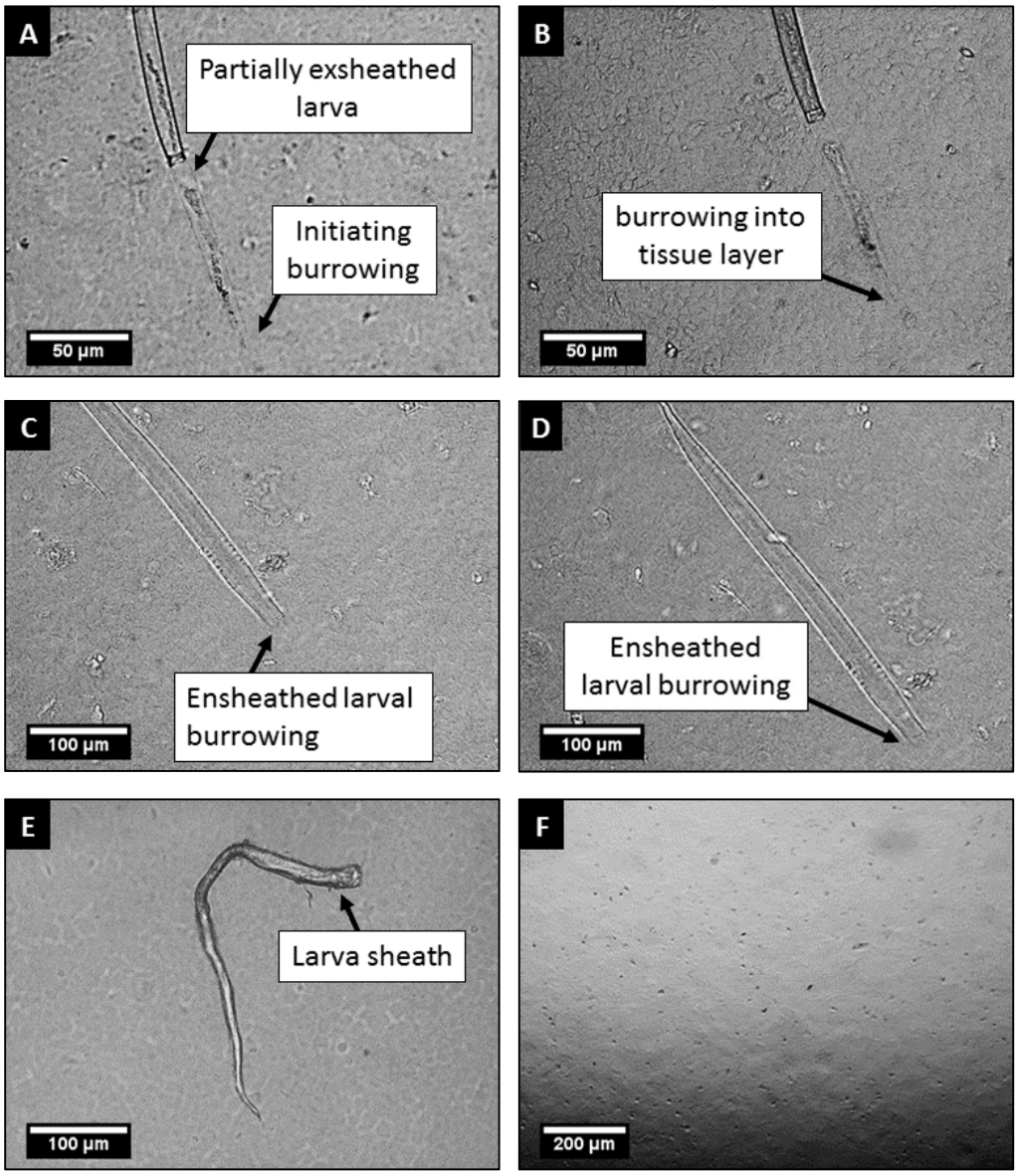


Figure 48: ***Necator americanus*** **interaction with HaCat cells**.

Panels A and B illustrate the capability of partially exsheathed larvae burrowing into the HaCat cell layer. In contrast panels C and D demonstrate ensheathed larval burrowing. There were only a few larvae sheaths identified on the apical surface of the HaCat cells (E), with the majority of larvae successfully burrowed ensheathed into the cell layer and no longer present on the apical surface (F). Data shown are representative of 3 independent experiments and 3 larvae batches.

4.1.2 The effect of *Necator americanus* on epidermal keratinocytes (HaCat) viability and morphology

Upon contact with the HaCat cell layer, *N. americanus* larvae interact with lipid receptors prompting the release of enzymatic secretions which digest skin macromolecules (Brown, Girod et al. 1999, Loukas and Prociv 2001). Previous *in vitro* studies have observed the L_3 larvae to penetrate the epidermal and dermal layers of human skin, with a distinct passage of cellular destruction (Matthews 1982). Therefore, cell morphology and viability of the HaCat cells was investigated in order to assess the extent of cell damage caused by the *N. americanus* larvae, for a period of 72 hours. In parallel untreated HaCat cells were imaged to establish a control for healthy morphology. The data illustrated that cells cultured with the larvae exhibited an abnormal phenotype signifying tissue damage (Figure 49). The HaCat cells appeared to be digested and ruptured, progressively deteriorating in cell morphology, with darkened areas and necrotic-like craters forming across the tissue layer. Although the morphology indicated cell death, it is important to highlight that the described changes were quite local and seem to be limited to the point of direct physical contact between the larvae and HaCat cell layer.

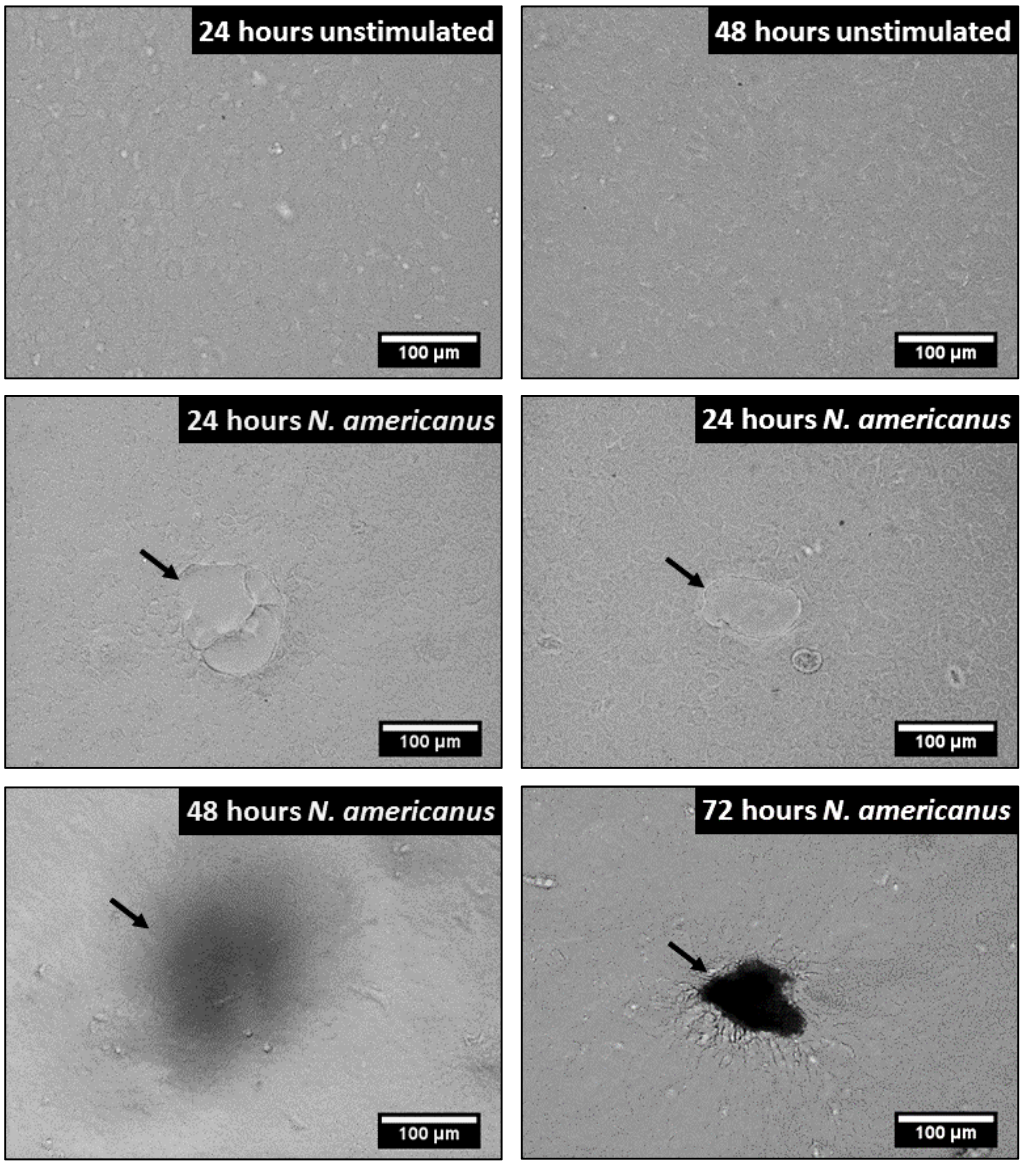


Figure 49: The morphology of *Necator americanus* treated HaCat cells.

The untreated HaCat cells were grown to confluency and exhibited no signs which could indicate an infection or cellular damage at both 24 and 48 hours. However, post incubation with *N. americanus*, the cells developed an abnormal phenotype, with tissue digestion, rupture and necrosis, at the site of larvae contact. Data shown are representative of 3 independent experiments.

Subsequently, the extent of *N. americanus* cytotoxicity on HaCat cell viability was examined. The cells were incubated with 50 L_3 *N. americanus* larvae for 24 and 72 hours, and were then stained with the Annexin V cytotoxicity assay. Despite apparent local impact of cell viability at the point of contact between the larvae and HaCat cell layer, the overall percentages of cells staining negative for Annexin and PI were comparable to untreated cells. However there was a slight increase in the number of necrotic cells (Annexin V^+/PI^+) in *N. americanus* treated HaCat cells at the 72 hour time point (Figures 50 and 51).

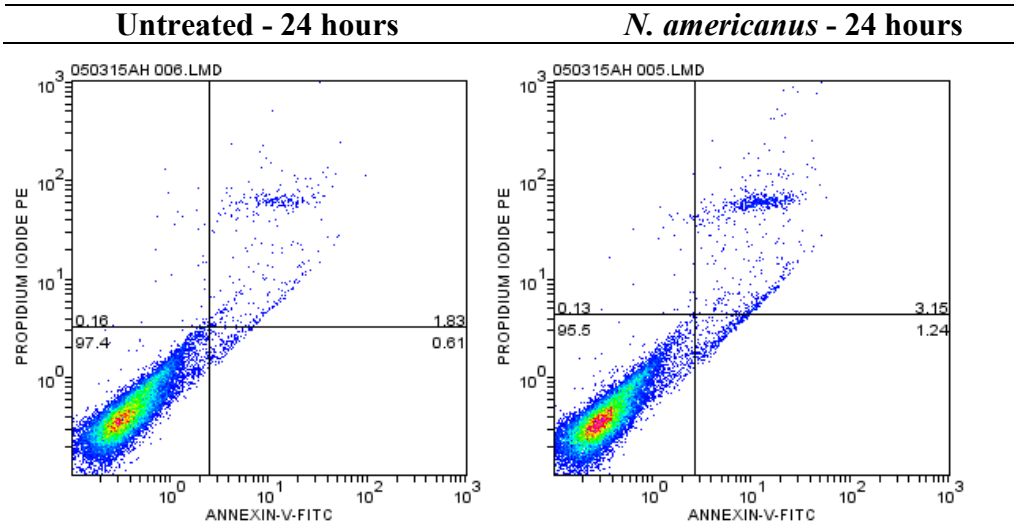


Figure 50: The viability of HaCat cells 24 hours post treatment with *Necator americanus* larvae.

HaCat cells remained viable at 24 hours post infection with the infected cells staining 95%≥ negative for Annexin V and PI. Data shown are representative of 3 independent experiments and 3 larvae batches.

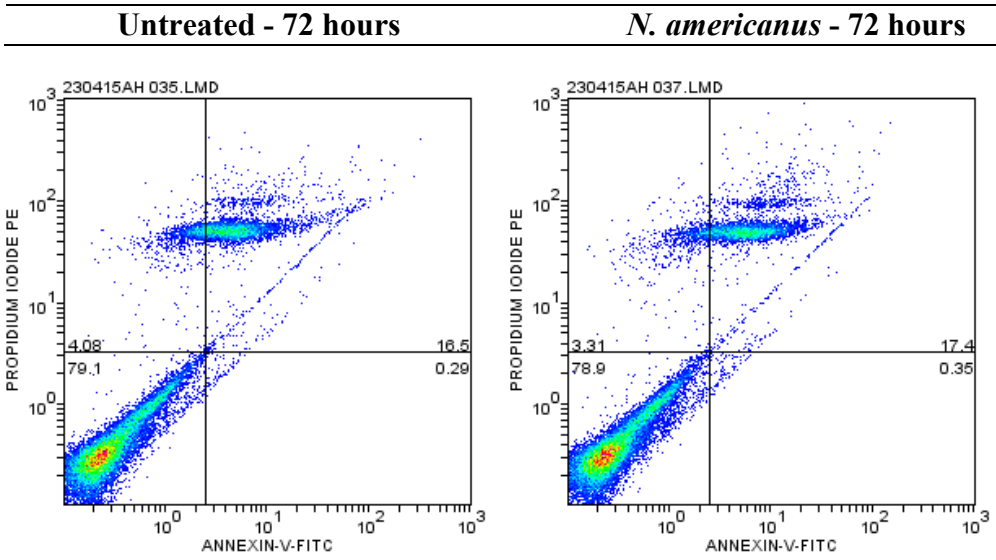


Figure 51: The viability of HaCat cells 72 hours post treatment with *Necator americanus* larvae.

HaCat cells remained viable at 72 hours post infection with the infected cells staining 78%≥ negative for Annexin V and PI. Data shown are representative of 3 independent experiments and 3 larvae batches.

4.1.3 IL-8 expression by epidermal keratinocytes (HaCat) in response to *Necator americanus*

The expression of the chemokine IL-8, primarily functioning to stimulate chemotaxis and vascular permeability (Waugh and Wilson 2008), was investigated in HaCat cells co-cultured with L_3 *N. americanus* larvae. The cells were grown to confluency and incubated with approximately 50 ensheathed *americanus* larvae, from which the supernatant samples were collected at 24 and 72 hours. Intriguingly, IL-8 expression in infected HaCat cells was comparable to expression by unstimulated cells. In parallel cells stimulated with LPS as a

positive control expressed significantly higher levels of IL-8 at both 24 and 72 hours (Figure 52).

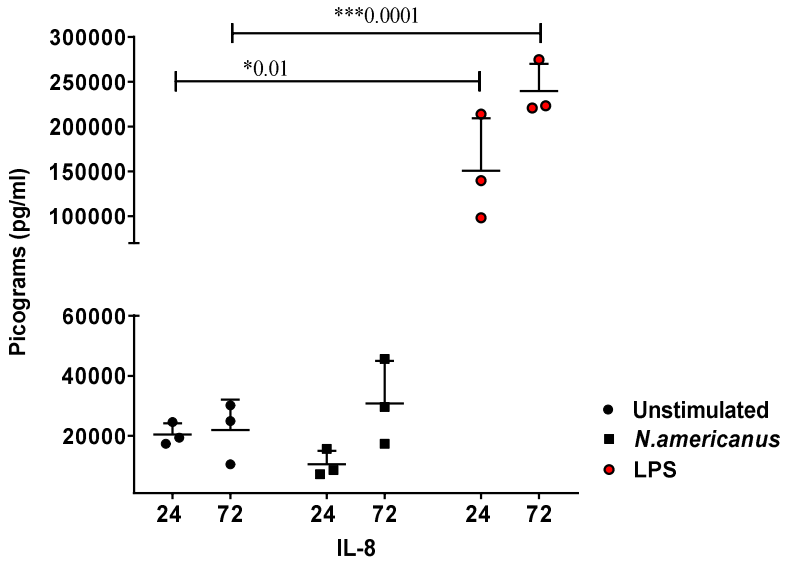


Figure 52: The cytokine expression of IL-8 by *Necator americanus* or LPS stimulated HaCat cells (± SEM).

In response to LPS, HaCat cells expressed significantly higher level of IL-8 in comparison to expression by unstimulated or *N. americanus* treated cells ($p = 0.001$ at 24 hours and $p = 0.0001$ at 72 hours respectively). Data shown are representative of 3 independent experiments.

4.2 Discussion

4.2.1 The immunological benefit of ensheathed larval burrowing

The localization of discarded *N. americanus* sheaths have been studied *in vivo*, in neonatally infected hamsters, examining the life cycle, growth and migration of larvae (Behnke, Paul et al. 1986). These studies reported that the larvae sheaths do not accompany the hookworms post the skin stages of infection (i.e., in the lungs, gastrointestinal tract). This was confirmed by examining larvae recovered from the lungs, which were exsheathed and developmentally enhanced, signifying the lung mediated stage of Necatoriasis (Behnke, Paul et al. 1986). Furthermore, the larvae recovered from the intestine were significantly longer, exhibiting hallmark characteristics of mature hookworms (i.e. full buccal capsule, cutting plates etc.). Intriguingly, these studies also reported that following the initial infection, larvae remained dormant in the skin tissue for 48 hours, with migration to the lungs commencing at 72 hours (Behnke, Paul et al. 1986). Other early research in *in vivo* mice experiments, reported that the majority of deposited larvae were penetrating the skin without exsheathing. This was evident by the recovery of only a few larval sheaths on the outer skin surface following infection (Kumar and Pritchard 1992). Interestingly the data presented in Chapter 4 (Figure 48) concur. Albeit some larvae exsheathed prior to penetrating the intact HaCat cell layer, the majority had burrowed ensheathed. Overall these data allude to a possible preference of larvae maintaining their sheaths during skin penetration, which in theory would be highly beneficial to

the *N. americanus*. The advantage of larvae maintaining their sheath during the initial stages of infection complements the data presented in Chapter 3, as the sheath is necessary for DC sequestration.

Furthermore, the data also illustrates the novel, distinctly modified behaviour of *N. americanus* larvae in culture with HaCat cells (Figures 44-47). In terms of exsheathing, the larvae were notably lethargic and slower to discard their sheath in culture with HaCat cells. In addition, the larvae remained in a state of resting posture, exhibiting no dynamic movements, as observed in DC cultures. The most plausible justification for this sudden dormancy in larval behaviour, is that in order to elicit a dynamic physical response from the hookworm, an external stimuli is required. Early research on the behaviour of *N. americanus* larvae, illustrated that prior to contact with the host, larvae are generally stationary, in a continuous state of energy preservation (Haas, Haberl et al. 2004). The larvae only reversed this latent posture, following the detection of signals such as vibration and touch. In this context, the epidermal keratinocytes function as an anatomical barrier; contrasting the APC capabilities of DCs, migrating to and physically interacting with the larvae, consequently prompting a behavioural response.

However, referring to the data presented in Chapter 3, the excessive exsheathing of *N. americanus* larvae requires a mandatory interaction with both MR and DC-SIGN (Figure 22). Keratinocytes express a range of PRRs including TLR2, TLR5, but more importantly the keratinocyte mannose binding receptor (KcMR)

(Pivarcsi, Kemeny et al. 2004). The KcMR expressed by HaCat cells would hypothetically interact with the MR ligands present on the larvae sheath, during larval penetration of the keratinocyte layer. Notably, DCs treated with only α-DC-SIGN (with the MR still available for interaction) were still capable of binding the larvae sheath, albeit at decreased levels. Although DCs were binding through via the MR ligands present on the larvae sheath, this did not stimulate exsheathing (Figure 22). Arguably, *N. americanus* larvae require interactions with both MR and DC-SIGN in order to induce excessive exsheathing, the latter of which is not expressed on HaCat cells. Furthermore, in addition to the absence of specific CLRs, there is a possibility that the mediators necessary for stimulating exsheathing, are not present in the keratinocyte cytokine microenvironment, therefore further promoting ensheathed larval burrowing into the tissue layer.

Continuing, the period of larvae dormancy in the skin mediated stages of infected animal models (Behnke, Paul et al. 1986), concur with the data presented in Figure 47, illustrating prolonged hookworm inactivity. Considering that the larvae are well adapted and employ behavioural strategies promoting host invasion (Haas, Haberl et al. 2004); this delay in the skin could be another hookworm strategy. For instance, following the detection of specific stimuli such as movement and heat emitted by the target host, a hookworm invasion strategy is initiated (Schad, Chowdhury et al. 1973, Pritchard 1995). The *N. americanus* larvae arrange themselves, become erected and their anterior ends move in multiple ways, a process referred to as nictating or 'searching and waving'

(Haas, Haberl et al. 2004). Rationally, the observed period of inactivity in the skin, could be an immune diversion strategy, enabling time for immune cell migration to the site of infection, and as shown in Chapter 3 (Figures 19 and 20), sequester of DCs onto the larval cuticle sheath.

Consequently based on the collective findings in Chapters 3 and 4, the biological relevance of the heavily glycosylated larvae sheath, appears to be tailored for bypassing the innate immune cells as opposed to the anatomical barrier. It is a fundamental feature for immune evasion, ensuring parasite survival. However the validity of such speculation is yet to be investigated using appropriate *in vivo* models.

4.2.2 The mechanism of *Necator americanus* skin penetration

The mechanical burrowing of *N. americanus* larvae has been associated with lipids present on the skin. The removal of chloroform soluble lipids *in vitro* human skin studies significantly decreased the number of larvae penetrating the epidermal and dermal layers (Matthews 1982). This study also reported enzyme secretions by the larvae, detected through an azocoll protease assay, with peak activity at 37 ^{0}C. The study suggested larval burrowing stages to be divided into two segments; primarily the mechanical burrowing phase of the epidermis, and the functional enzymatic secretions, which enable navigation through the germinal layers and vascular endothelium (Matthews 1982). Studies have shown

that larval proteases have been shown to increase endothelial permeability, linked to an overexpression of IL-6/IL-8, in addition to the disruption of vascular endothelial cadherin and F-actin in umbilical vein endothelial cells (Souadkia, Brown et al. 2010). Intriguingly, IL-8 levels in HaCat cells infected with the larvae did not increase, albeit the morphology indicating tissue damage. The deterioration observed was local and at the site of physical interactions, marked by darkened, ruptured and necrotic-like areas on the cell surface.

Infection with *N. americanus* is associated with a variety of symptoms including a local inflammation, redness and itching (Feary, Venn et al. 2010, Liu and Cao 2016, Loukas, Hotez et al. 2016) which was thought to be a major contributing factor enabling larval penetration of the skin layer. It is essential to highlight that the expression of inflammatory mediators such as IL-8, would have elicited an increased permeability of the skin barrier (Waugh and Wilson 2008), which when intact permits the passage of molecules ≤40 (Brandner, Zorn-Kruppa et al. 2015). The lack of cytokine expression was not due to a decrease in cell viability, confirmed with the Annexin cytotoxicity assay. It would therefore seem likely that in the absence of inflammatory cytokines, both the mechanical aspect of larval burrowing into the HaCat cells layer, combined with skin proteases secreted by the larvae (Matthews 1982, Kumar and Pritchard 1992, Loukas and Prociv 2001) are the predominant factors enabling penetration through the epidermal layers of the skin.

To date there are only a few studies (*in vitro*), which have investigated the interactions between *N. americanus* and components of the human epidermis. These reported that the larvae can penetrate the human skin from epidermal and dermal directions, marked by a passage of distinct cellular destruction at the site of larvae contact (Matthews 1982). These findings concur with data presented in Chapter 4 (Figures 9) as *N. americanus* infected HaCat cells also deteriorated in morphology. The tissue damage observed is plausibly due to larval secretions, which have been shown to contain hydrolysing aspartyl proteinases. Albeit the validity of such speculations requiring further research, larval proteases degrade ECM components of the skin including collagen, elastin, fibronectin and laminin (Brown, Girod et al. 1999). More importantly, the inhibition of these gelatinolytic proteinase secretions via pepstatin-A, prevents larval burrowing (Brown, Girod et al. 1999), establishing a fundamental role of the aspartyl proteinases in skin penetration.

4.2.3 Conclusion and future work

There was a significantly limited source of larvae availability for this project, which affected the range of experimental work conducted. However, collectively, the data obtained provide new insights into the modified behaviour of *N. americanus* larvae and the potential invasion strategy employed to promote parasite survival. It is therefore paramount to analyse and compare a microarray of known keratinocyte ligands on the larvae sheath, which can identify further receptor-ligand interactions. For instance, blocking HaCat cells with Mannan or

α-MR antibodies, prior to larval incubation, will provide further insight into the role of MR ligands on the larvae sheath during skin penetration.

Furthermore, larval burrowing into the skin layer could be aided by the presence of LPS (*in vivo*), leading to an increase in early inflammatory mediators i.e. IL-8 and TNF-α. An increase in chemokines could explain the entry gained by the hookworm larvae into the skin. However the larvae employed in this study were axenised, which is not the case *in vivo* (Loukas and Prociv 2001). It is therefore vital to assess the cytokine expression and morphology of HaCat cells following incubation with un-sterile hookworm larvae.

In addition, it is imperative to note that *in vivo* (i.e., in the skin), there are stromal cells such as dermal fibroblasts present, which could alter the secreted cytokines/chemokines in the microenvironment. The dermal fibroblasts secrete a range of cytokines essential for cellular adhesion growth, stability and organization of the ECM during wound healing (Kubo and Kuroyanagi 2005, Tracy, Minasian et al. 2016). This could significantly affect larvae behaviour, providing a basis for further research, focused on a biologically relevant model. Therefore a 3D culture system is presented in Chapter 5, encompassing dermal fibroblasts (BJ-6), epidermal keratinocytes (HaCat) and DCs, in a relevant spatial orientation.

CHAPTER 5: The development of a 3D culture system encompassing keratinocytes, fibroblasts and dendritic cells to study hookworm infection

5.1 Results

Albeit the human skin acts as a multifunctional defence barrier against physical, chemical and microbial factors (Bouwstra, Honeywell-Nguyen et al. 2003, Grice and Segre 2011); it is ineffective in the prevention of *N. americanus* larvae penetration (Matthews 1982). However, no such interactions have been studied extensively *in vivo*, as the majority of animal models develop atypical traits in comparison to a human hookworm infection (Loukas and Prociv 2001). This renders their biological relevance unrepresentative, particularly with regards to skin penetration and interaction with innate immune cells, such as DCs within the skin (Miranda de Carvalho, Bonnefont-Rebeix et al. 2006). Additionally, the ethical pressure to abolish the use of animals for testing (Adler, Basketter et al. 2011), further highlights the need for an alternative and physiologically applicable experimental platform.

Consequently, to better simulate early events at the interface of larvae and human skin, the aim of the work described in Chapter 5 was to establish an immunocompetent 3 dimensional tri-culture system. This skin model is based on and adapted from previous studies (Chau, Johnson et al. 2013). The tri-culture encompasses epidermal keratinocytes (HaCat) and dermal fibroblasts (BJ-6), interspersed with DCs cultured at air liquid interface (ALI). In this chapter an effective skin barrier was optimized and examined, following which immune cell migration within the tri-culture system was observed successfully. However,

due to the inconsistent and limited source of larvae availability (Tanasescu and Constantinescu 2015), the infection of the skin model was only attempted.

5.1.1 The development of a PET scaffold for tissue culture

In order to provide a suitable scaffold support for the cellular components of the 3D tri-culture system, PET was developed by electrospinning as described, following established protocols within the group (Harrington, Cato et al. 2014). The highly porous interconnected nature of the PET scaffold fibres, facilitate cellular growth, interaction between different components in the tri-culture, and the migration of immune cells (Harrington, Cato et al. 2014).

The SEM micrograph of the scaffold (Figure 53) illustrated an average fibre diameter of 0.385 μm (+0.275/-0.185), which has been shown to support fibroblast and epithelial cell growth and differentiation (Chau, Johnson et al. 2013, Harrington, Cato et al. 2014). Therefore, these scaffolds provided an effective platform for developing a tri-culture, with robust properties permitting long-term cultures, and the separation of tissue layers for further analysis of individual cell types (Figure 53).

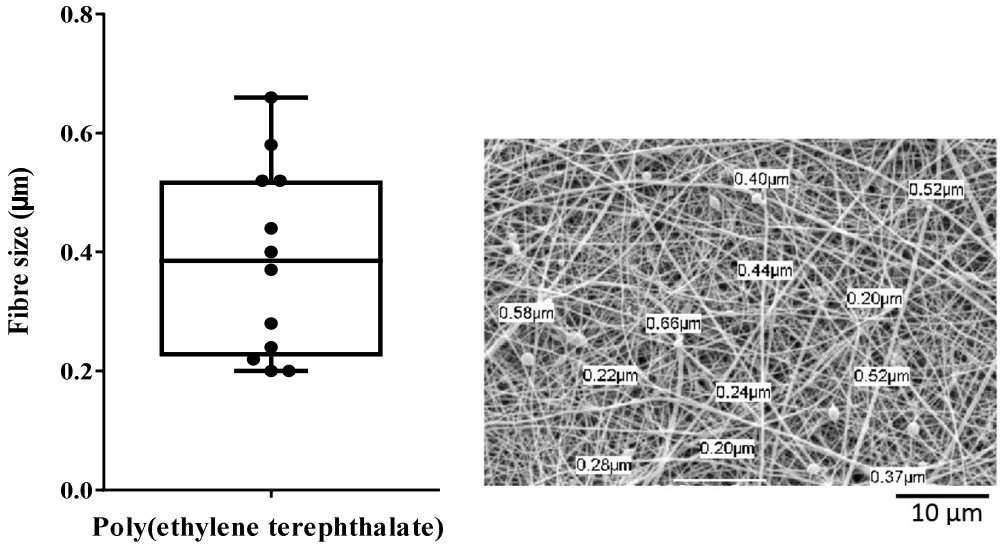


Figure 53: Scanning electron micrograph and fibre diameters of PET scaffold (± SEM).

The PET scaffold has a mean fibre diameter of 0.385 µm (+0.275/-0.185). The scaffold has a highly porous interconnected structure which serves to provide a suitable microenvironment supporting cellular growth. Data shown are representative of 3 independent experiments.

5.1.2 Characterising the development of a functional barrier in epidermal keratinocytes (HaCat)

5.1.2.1 Single culture barrier formation

The HaCat cells incorporated into the tri-culture were used as a surrogate for human primary keratinocytes. In order to construct an *in vitro* model of the human epidermis in an immunocompetent tri-culture, it was crucial to ensure the development of a functional barrier in the HaCat cells. Barrier formation was confirmed by measuring transepithelial electrical resistance (TEERs), the

uniform expression of tight junction protein Zonula occludens (ZO-1) via confocal microscopy, and the development of a homogenous surface layer of extracellular matrix (ECM) connective tissue fibres via SEM analysis. The TEER values and ZO-1 expression were investigated in single culture models, cultured on polycarbonate transwell inserts (0.4µm pore size) to identify the optimum conditions for an effective barrier formation.

The results show that single cultures of HaCat cells at ALI on transwell inserts, for a period of 14 days, do not increase significantly in TEER values (maximum ~168 Ω cm^2). However HaCat cells stimulated with TGF-β (2 ng/ml) and GM-CSF (50 ng/ml), developed higher TEER values, with a peak/plateau 14 days at ALI (maximum ~232 Ω cm^2). In parallel the TEER values from acellular (blank) PET scaffolds and BJ-6 single cultures were measured as a control (maximum ~88 and 134 Ω cm^2 respectively) (Figure 54).

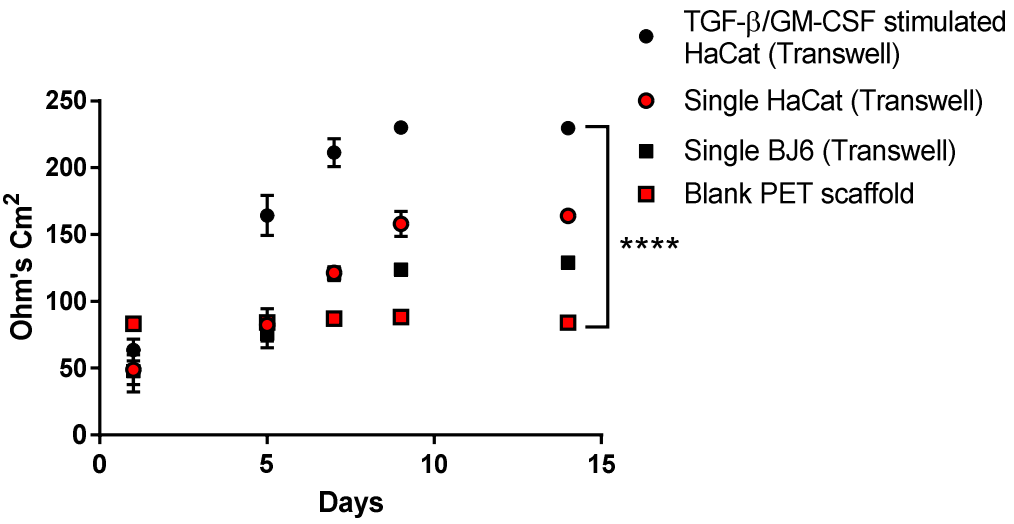


Figure 54: The TEER measurements of single HaCat cell cultures at ALI (± SEM).

The TEER values measured in single cultures of HaCat cells stimulated with TGF-β (2 ng/ml) and GM-CSF (50 ng/ml) are significantly higher (p value ≤ 0.0001) than unstimulated single HaCat cultures. In addition low TEER values were recorded for the acellular (blank) PET scaffolds and BJ6 cultures as a control. Data shown are representative of 3 independent experiments.

Subsequently, the single cultures of HaCat cells, were then evaluated for the expression of ZO-1 tight junction protein. The transwell insert layers were stained with Hoechst 33342 (nuclear stain) and ZO-1 antibody in order to identify the localization and expression of this protein in HaCat cells. The development of ZO-1 was assessed on days 7 and 14 at ALI in TGF-β/GM-CSF stimulated HaCat cells (Figures 55 and 56). In the unstimulated cells, ZO-1 expression was only assessed 14 days at ALI (Figure 57). In parallel, as a negative control, HaCat cells were grown on polycarbonate transwell inserts and stimulated with TGF-β/GM-CSF. These were then assessed on day 14 and stained with only the secondary antibody for ZO-1, conjugated to a fluorescent dye (Figure 58).

The results illustrate that HaCat cells cultured at ALI for a period of 7 days in the presence of growth factor cytokines, have a low and uneven expression of ZO-1 across the cell layer (Figure 55). However, following 14 days at ALI, HaCat cells expressed a uniform layer of the tight junction protein (Figure 56). In contrast, unstimulated single cultures failed to develop ZO-1 at 14 days ALI, corresponding with the low TEER values recorded (Figures 57). Finally, there was no unspecific binding of ZO-1 tight junction protein, evident by the lack of

fluorescence detected in TGF-β/GM-CSF stimulated HaCat cells, stained only with the secondary antibody for ZO-1 (Figure 58).

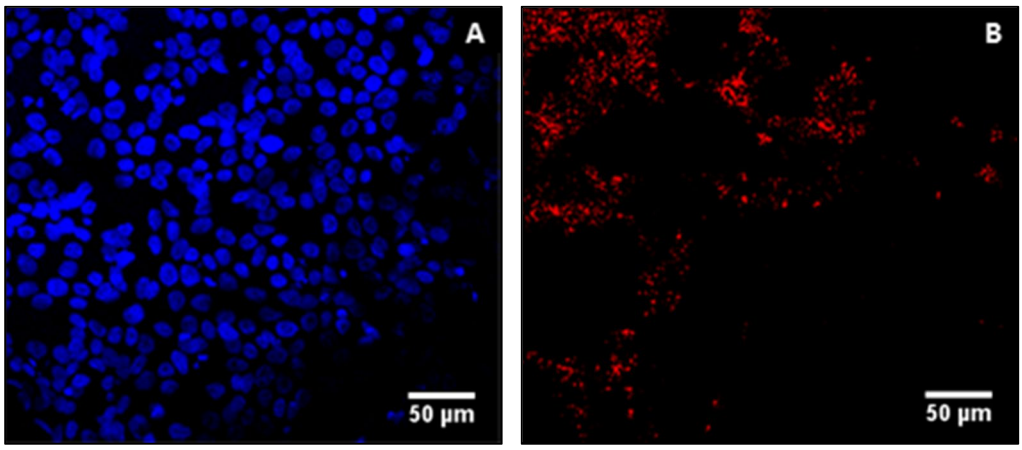


Figure 55: Single HaCat cell culture stimulated with TGF-β/GM-CSF on 0.4 µm pore polycarbonate membrane inserts at 7 days ALI.

Hoechst nuclear stain (A) and ZO-1 (B) illustrated separately. Following 7 days at ALI, ZO-1 expression in HaCat cells stimulated with TGF-β and GM-CSF was low and uneven across the cell layer. Data shown are representative of 3 independent experiments

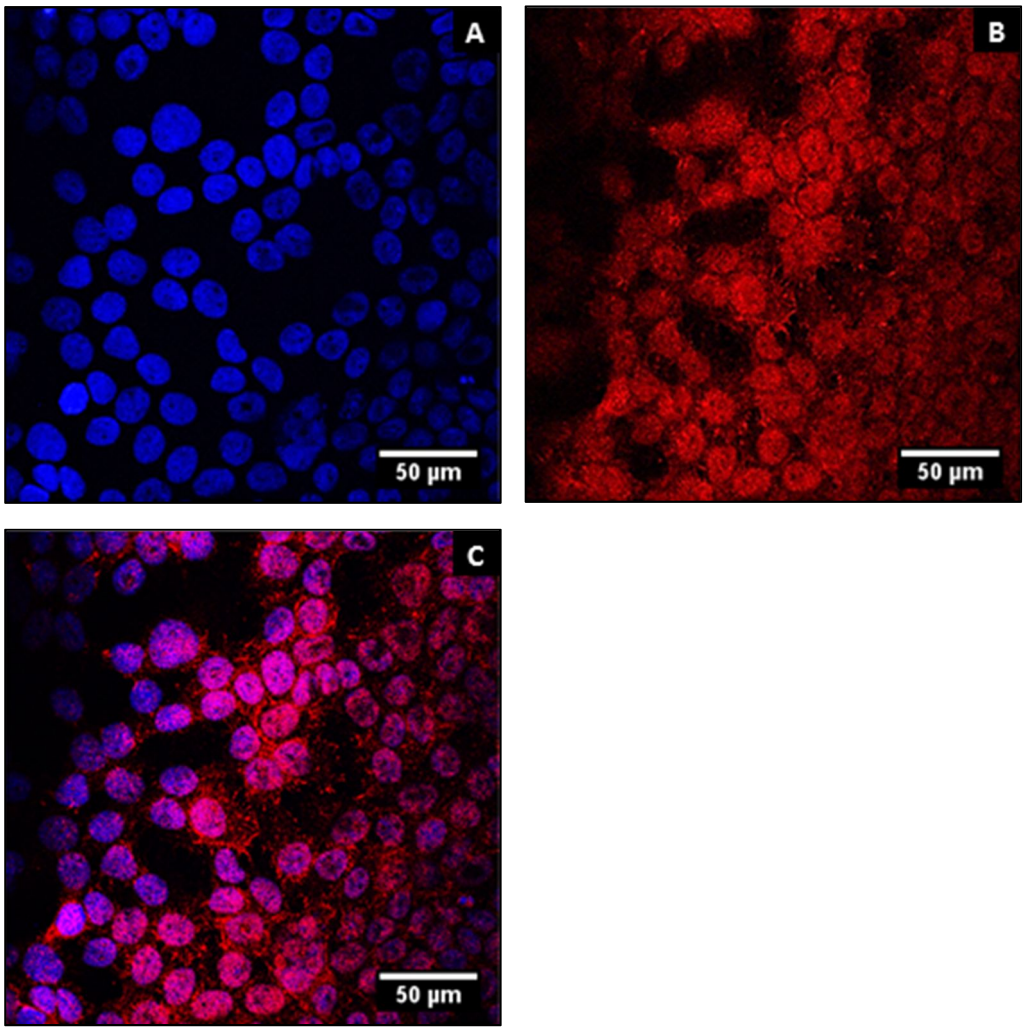


Figure 56: Single HaCat cell culture stimulated with TGF-β/GM-CSF on 0.4 µm pore polycarbonate membrane inserts at 14 days ALI.

Hoechst nuclear stain (A), ZO-1 (B) and the overlay (C) illustrated separately. Following 14 days at ALI, ZO-1 expression in HaCat cells stimulated with TGF-β and GM-CSF was uniform across the cell layer. Data shown are representative of 3 independent experiments.

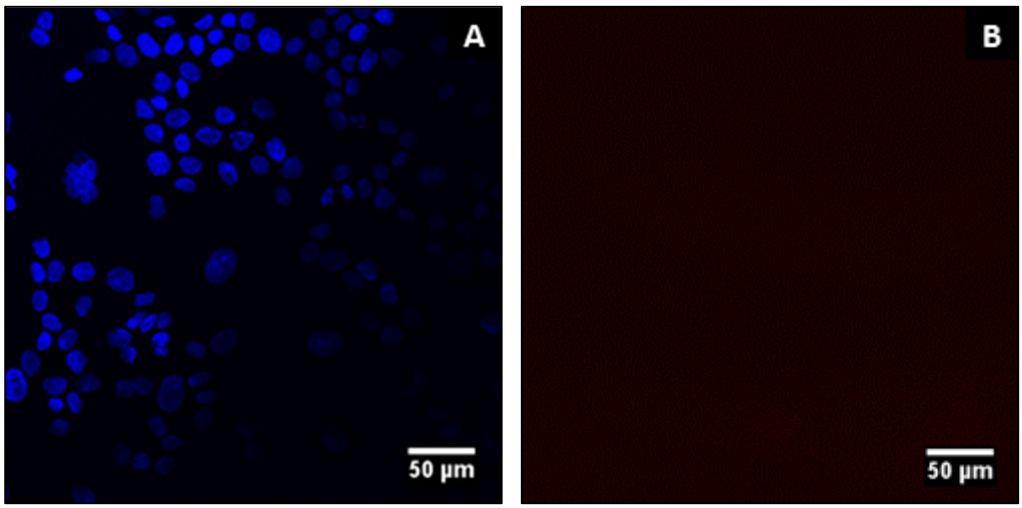


Figure 57: Unstimulated single HaCat cell culture on 0.4 µm pore polycarbonate membrane inserts at 14 days ALI.

Hoechst (A) and ZO-1 (B) illustrated separately. Following 14 days at ALI, the tight junction protein ZO-1 was not detected/expressed in unstimulated HaCat cells. Data shown are representative of 3 independent experiments

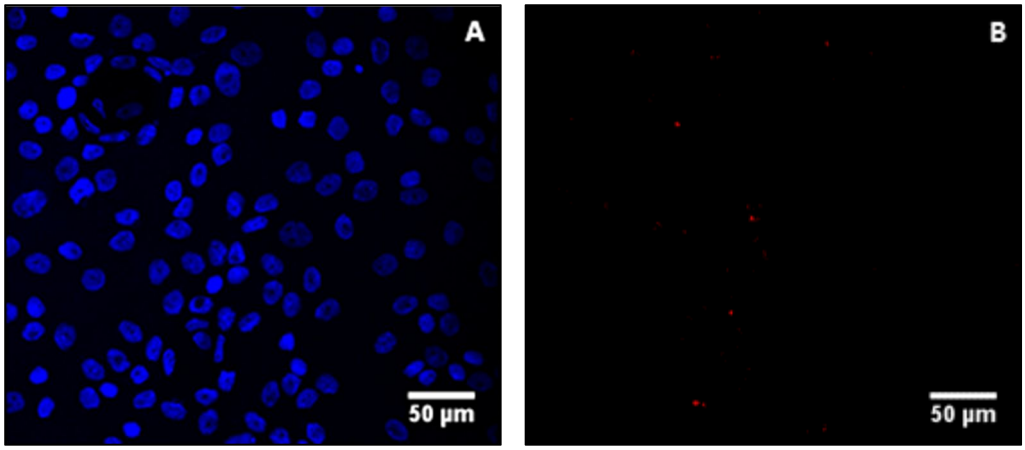


Figure 58: Single HaCat cell culture stimulated with TGF-β/GM-CSF on 0.4 µm pore polycarbonate membrane inserts at 14 days ALI.

Hoechst (A) and secondary antibody for Z0-1 conjugated to a fluorescent dye (B) illustrated separately. There was no unspecific binding of ZO-1 in stimulated single cultures of HaCat cells at 14 days ALI. Data shown are representative of 3 independent experiments.

5.1.2.2 Co-culture barrier formation

The TEER values and ZO-1 expression were subsequently investigated in co-culture models (HaCat and BJ-6 cells) on PET scaffold or polycarbonate transwell inserts (0.4µm pore size). This was conducted to identify the optimum conditions necessary for the development of an effective barrier. The results show that HaCat cells grown in co-cultures with BJ-6 at ALI, on PET or transwell inserts, developed increased TEER values, which peaked/plateaued 14 days at ALI (maximum ~266 and 236 Ω cm respectively). In parallel the TEER values from acellular (blank) PET scaffolds and BJ-6 single cultures were measured as a control (maximum ~88 and 134 Ω cm respectively) (Figure 59).

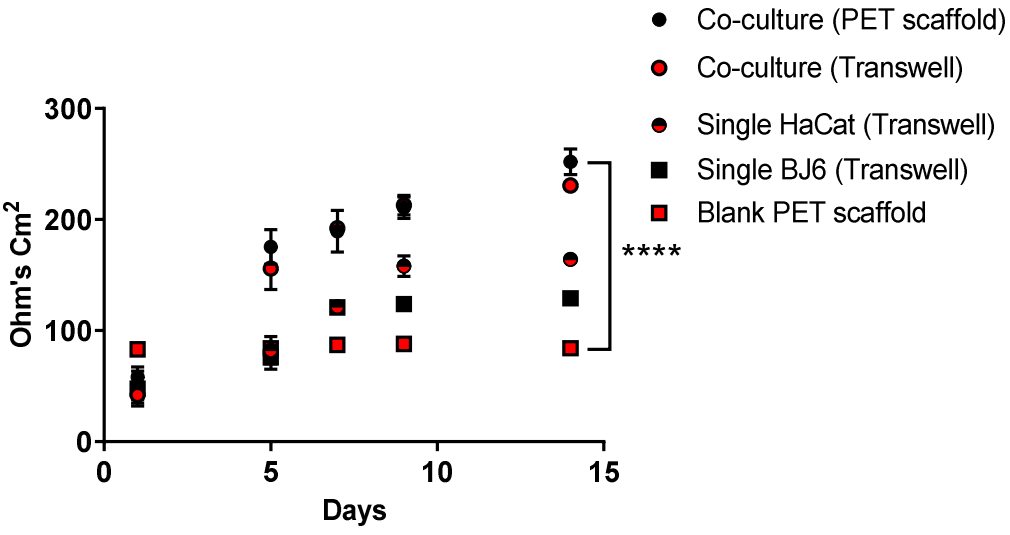


Figure 59: The TEER measurements of co-cultured HaCat and BJ-6 cells at ALI (± SEM).

The TEER values measured in co-cultures of HaCat/BJ-6 cells (PET or polycarbonate transwell) are significantly higher (p value ≤ 0.0001) than unstimulated single HaCat cultures. In addition low TEER values were recorded

for the acellular (blank) PET and single BJ6 cultures as a control. Data shown are representative of 3 independent experiments

Subsequently, the HaCat cell layer from co-cultures grown on PET or polycarbonate transwell inserts, were evaluated for the expression of ZO-1 tight junction protein. The scaffold and transwell layers were stained with Hoechst 33342 (nuclear stain) and ZO-1 antibody in order to identify the localization and expression of this protein in HaCat cells. The development of ZO-1 was assessed on day 14 at ALI which illustrate a uniform expression of ZO-1 in PET and transwell co-cultures (Figures 60 and 61 respectively).

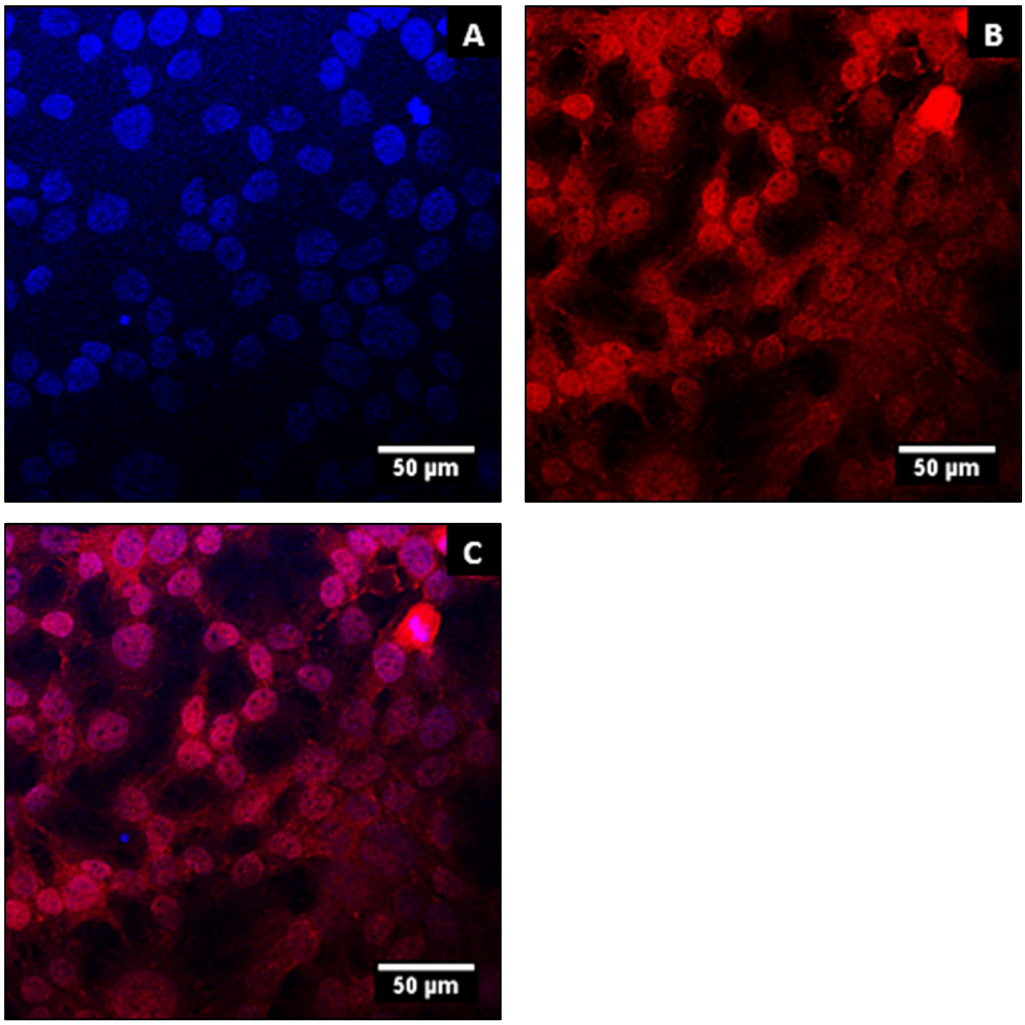


Figure 60: The HaCat cell layer from co-cultures with BJ-6 14 days at ALI on 0.4 µm pore polycarbonate membrane inserts.

Hoechst nuclear stain (A), ZO-1 (B) and the overlay (C) illustrated separately. Following 14 days at ALI, ZO-1 expression in HaCat cells from co-cultures was uniform across the cell layer. Data shown are representative of 3 independent experiments.

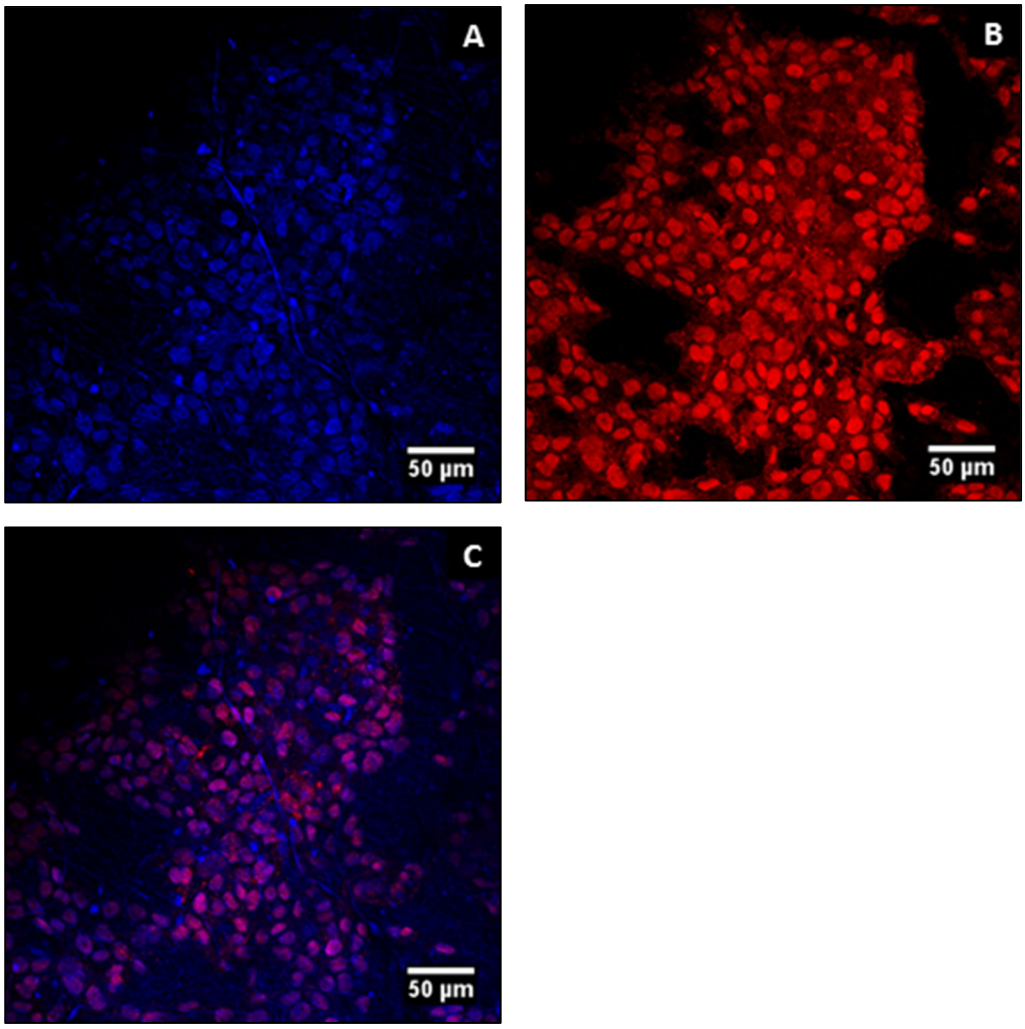


Figure 61: The HaCat cell layer from co-cultures with BJ-6 14 days at ALI on PET scaffold.

Hoechst nuclear stain (A), ZO-1 (B) and the overlay (C) illustrated separately. Following 14 days at ALI, ZO-1 expression in HaCat cells from co-cultures was uniform across the cell layer. Data shown are representative of 3 independent experiments.

5.1.2.3 The development of ECM in single and co-cultured HaCat cells

Following the data from TEER measurements and ZO-1 expression, single cultures of HaCat cells or co-cultures with BJ-6 cells were grown on PET scaffold. The cell-scaffold layers were evaluated for the development of a homogenous ECM layer, assessed on days 7 and 14 at ALI via SEM analysis.

The micrographs illustrated that single cultures of HaCat cells did not develop a confluent layer of ECM (Figure 62), in contrast co-cultures were optimal for ECM formation with homogenous layers observed at 14 days (Figure 63). In parallel the ECM formation in monolayers of BJ-6 cells was assessed as a positive control. Fibroblasts constitute the majority of cells in the dermis, synthesising the connective tissue fibres found within this layer and the ECM. These cells are crucial in the cellular adhesion, growth, stability and organization of the ECM. In addition the fibroblasts are essential for depositing a range of structural proteins and collagen into the ECM (Tracy, Minasian et al. 2016).

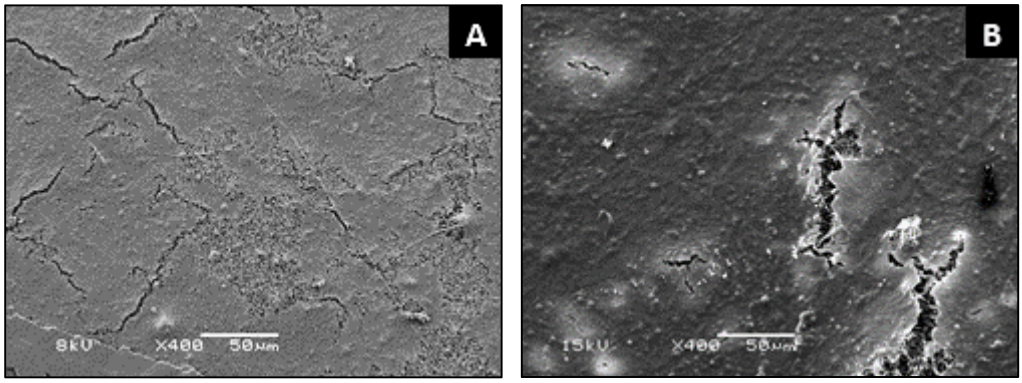


Figure 62: SEM micrographs illustrating ECM formation in single HaCat cell cultures on PET scaffold.

There is no homogenous ECM formation in single HaCat cell cultures on PET scaffold at ALI for 7 (A) and 14 (B) days. The magnification used to acquire the images was x400 and scale bars represent 50 µm. Data shown are representative of 3 independent experiments.

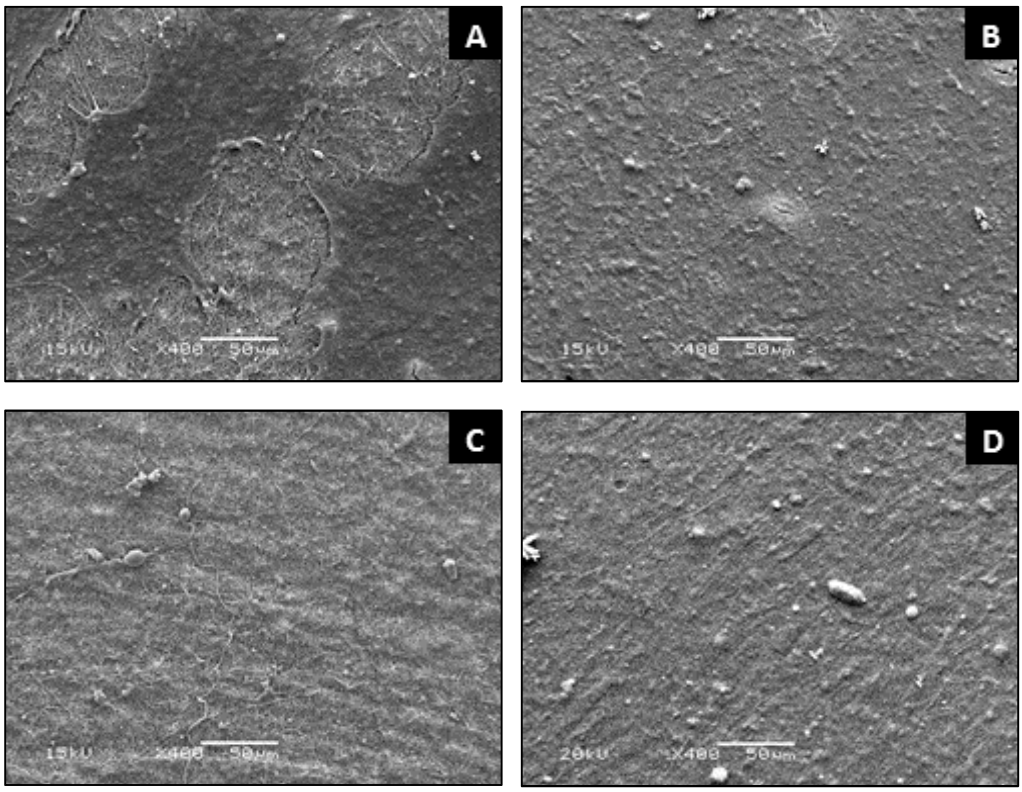


Figure 63: SEM micrographs illustrating ECM formation in co-cultures of HaCat and BJ-6 cells on PET scaffold.

There is no homogenous ECM formation in the HaCat layer from co-cultures at ALI for 7 days (A), however at 14 days (B) a uniform ECM layer is observed. On the BJ-6 scaffold from co-cultures, a homogenous ECM layer is formed at both 7 (C) and 14 (D) days at ALI. The magnification used to acquire the images was x400 and scale bars represent 50 μm. Data shown are representative of 3 independent experiments.

5.1.3 The assembly of an immunocompetent *in vitro* tri-culture model.

Co-cultures of differentiated HaCat with BJ-6 cells were cultured and assembled on PET scaffolds, held together in a scaffolder at ALI for 14 days. Subsequently, pre-stained (Hoechst) DCs seeded onto a PET scaffold were inserted between the HaCat and BJ-6 layers. The immunocompetent model is based on and optimised from previous studies (Chau, Johnson et al. 2013) where the top monolayer of the construct is comprised of fully differentiated epidermal keratinocytes (HaCat), the middle layer of monocyte derived dendritic cells (DCs) and finally the lower layer of dermal fibroblasts (BJ-6) (Chapter 2, Figure 16).

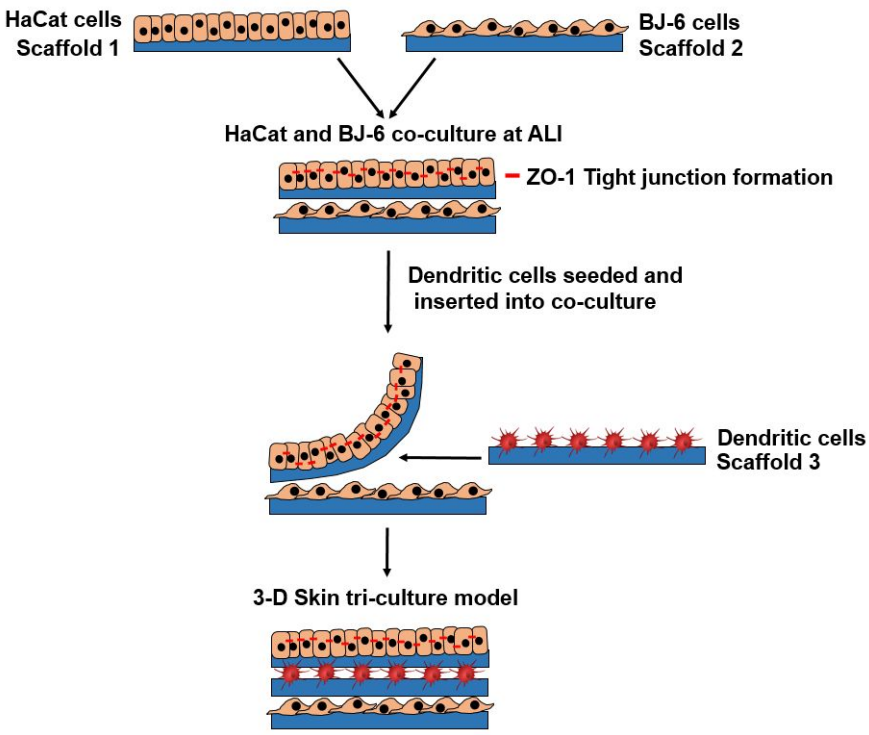


Figure 16: Schematic illustrating the stages of tissue-engineering a 3-D immunocompetent *in vitro* skin model.

The HaCat cells are seeded onto PET scaffold 1 and BJ-6 onto scaffold 2. These were co-cultured at ALI for 14 days in an in house engineered scaffholder (Harrington, Cato et al. 2014) to enable differentiation and ZO-1 tight junction development (37 ^{0}C, 5% CO_2). The upper (HaCat) layer is then briefly lifted to insert a separate PET scaffold containing DCs on top of the lower (BJ-6) scaffold layer. The HaCat scaffold layer is then placed on top of the DC scaffold layer, effectively encapsulating this in the middle and forming a tri-culture model.

5.1.3.1 Dendritic cell migration

The function of DCs directly depends on their ability to migrate to the site of infection (Martin-Fontecha, Lanzavecchia et al. 2009). Thus the migration of pre-stained (Hoechst) immature DCs was examined in two different tri-cultures.

Primarily, model (A) encompassing HaCat-DC-BJ-6 (Figure 66) and (B) where the HaCat and BJ-6 monolayers were substituted with acellular scaffolds (i.e., without cells) (Figure 65). Both constructs were stimulated with 100 ng LPS or PBS as a control, for 24 hours (37 ^{0}C, 5% CO_2). In parallel, pre-stained (Hoechst) DCs were seeded onto sterilised PET as a single culture for 24 hours, assessing the localization and migration of DCs within the scaffold fibres (Figure 64). Furthermore, the upper layer in model (A), was post-stained with pan-cytokeratin, an epithelial cell marker which identifies the expression of intermediate filament forming proteins, necessary for epithelial organization (Schweizer, Bowden et al. 2006, Bragulla and Homberger 2009).

The data show that in the absence of LPS stimulation, DCs in model (B) migrate predominantly towards the upper scaffold, with some migration towards the lower scaffold. This concurs with observations reported in the literature (Chau, Johnson et al. 2013). However, following LPS stimulation, migration towards the upper scaffold is increased, with no cells observed in the lower scaffold. This was apparent by the decrease of DCs within the middle scaffold, on which they were initially inoculated (Figure 65). In comparison, single culture DCs on PET, remain largely within their scaffold (Figure 64).

Untreated

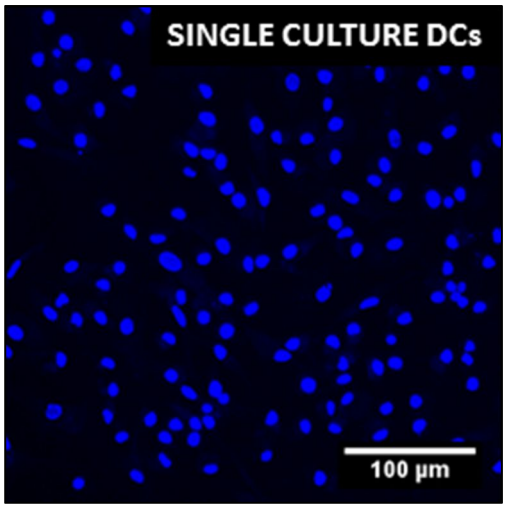


Figure 64: The migration of dendritic cells in a single culture on PET scaffold.

DCs were pre-stained with Hoechst (nuclear stain). The DCs cultured in the absence of LPS and further (upper/lower) scaffold layers, primarily remain within their scaffold and have an even distribution across the layer. Data shown are representative of 2 independent experiments.

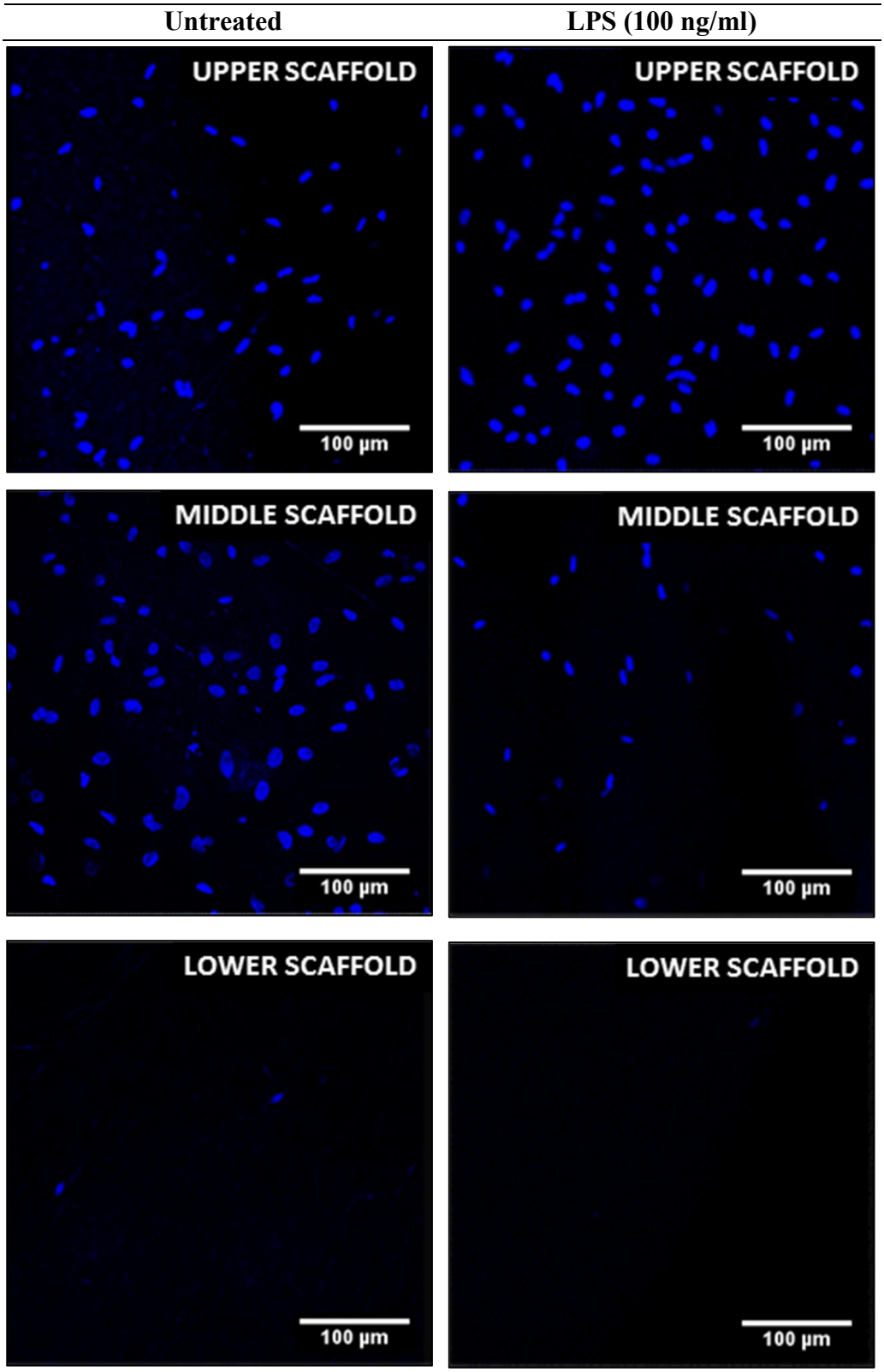


Figure 65: The migration of dendritic cells through an *in vitro* 3D PET model.

DCs were pre-stained with Hoechst (nuclear stain). In the unstimulated model, migration is predominantly towards the upper scaffold, with some migration towards the lower monolayer. However many cells remain within the middle scaffold where the cells were initially inoculated. In contrast following LPS stimulation, DC migration towards the upper scaffold is increased, with no cells detected in the lower scaffold. Data shown are representative of 2 independent experiments.

In contrast, unstimulated DCs in model (A) illustrated an increased migration to the lower (BJ-6) scaffold. However following LPS stimulation, these cells migrated predominantly to the upper (HaCat) scaffold, with some migration towards the lower (BJ-6) scaffold (Figure 66). Additionally, DCs in the middle scaffolds appear to be clustered together and notably larger than those observed in the lower (BJ-6) scaffold. Finally, there was a complete coverage and expression of pan-cytokeratin (green) across the upper (HaCat) scaffold, demonstrating epithelial organization (Bragulla and Homberger 2009).

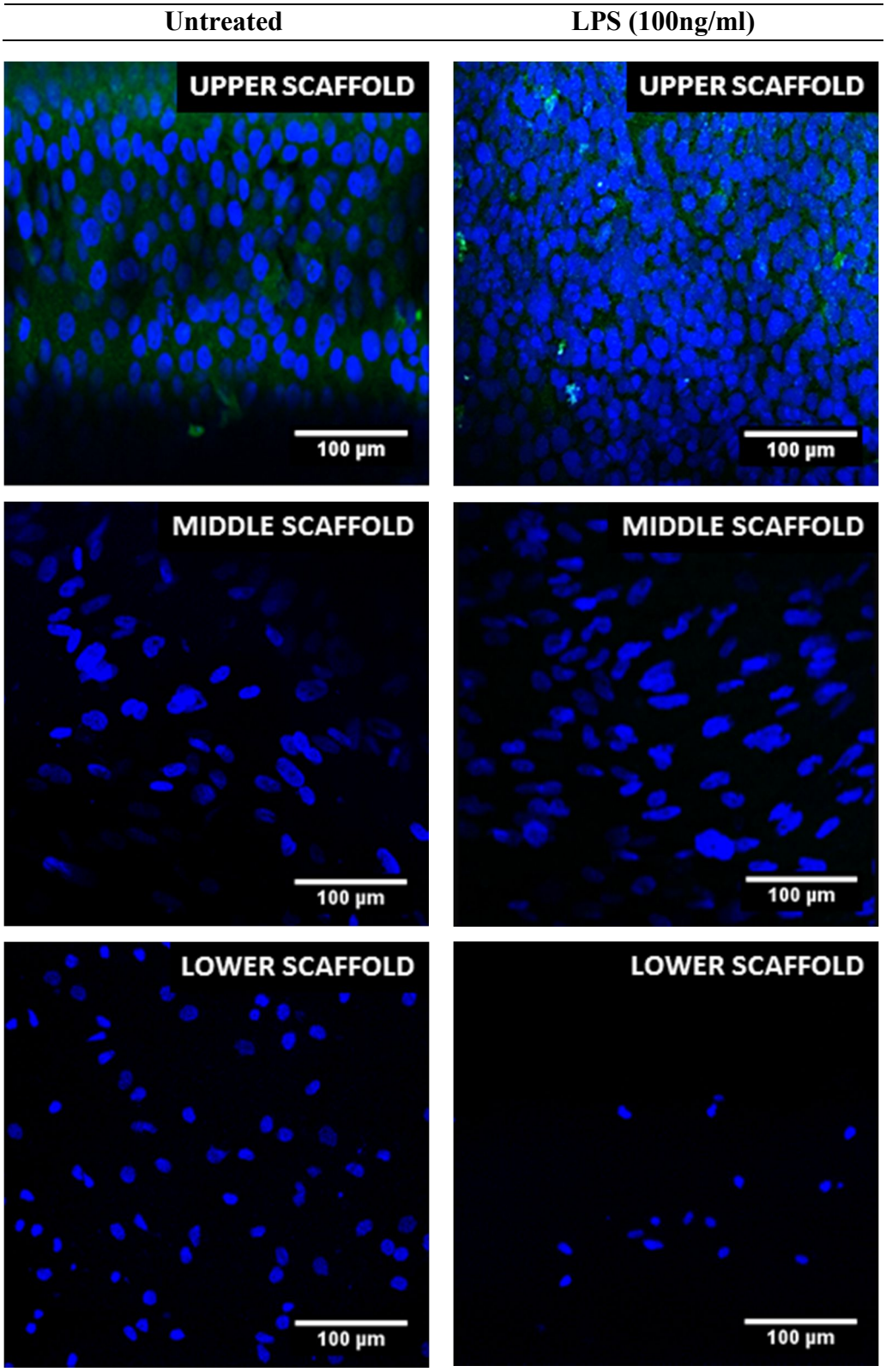


Figure 66: The migration of dendritic cells through an *in vitro* 3D tri-culture model.

DCs were pre-stained with Hoechst (nuclear stain) and HaCat cells were post-stained with pan-cytokeratin (epithelial marker). In the absence of LPS, the DCs

within the tri-culture model migrate primarily towards the lower (BJ-6) scaffold, with some remaining within the middle scaffold. However following LPS stimulation, DCs migrate predominantly towards the upper (HaCat) scaffold, with little migration towards the lower (BJ-6) monolayer. The DCs in the middle scaffolds appear to be clustered and the pan-cytokeratin stain on the upper (HaCat) layer was uniform. Data shown are representative of 2 independent experiments.

Collectively, these data clearly show that DCs are capable of migrating between the different layers of the construct, providing a proof of concept prior to infection with *N. americanus* larvae.

5.1.4 Exposure of the tri-culture to *Necator americanus* larvae

The data presented in chapter 3 (Figures 19/20) illustrated the interactions between infective *N. americanus* L_3 larvae and immature DCs on tissue culture treated plates. Rationally, prior to infecting the immunocompetent skin model, it was imperative to examine these interactions on the 3D PET scaffold fibres. These interactions were studied and captured with SEM analysis in order to visualize DC sequestration by the larvae sheath in more detail. To prepare this, DCs were seeded onto sterilized PET, pre-soaked in culture media as described, and allowed to settle for 24 hours in standard tissue culture conditions. Subsequently 50 *N. americanus* larvae were deposited onto the PET scaffold encompassing DCs; this was incubated for a further 24 hours. In parallel untreated scaffolds were also examined as a control. The scaffolds were fixed in 4% PAF and dehydrated accordingly for SEM analysis.

The micrographs clearly show that as observed in DC-larvae interactions on tissue culture plates, there were dense aggregates formed on the scaffold fibres in comparison to untreated PET (Figure 67). Additionally, there were no *N. americanus* larvae identified on the scaffold. Nonetheless, upon closer examination, the aggregates resembled the morphology of ECM depositions. Although the constituents of the aggregates remain to be elucidated, there were DCs identified, which were visibly attached/sequestered onto what appeared to be fragments of a discarded larvae sheaths (Figure 68). Notably, the fragments which were engulfed by DC aggregates were 20-50 µm in length, which is considerably smaller than the average *N. americanus* larva (300-400 µm). Arguably, unlike live interactions observed in Chapter 3 on tissue culture plates, the samples prepared for SEM analysis had undergone fixing (4% PAF) and extensive dehydration (0-100% ethanol) protocols. This could have possibly resulted in the fragmentation of the cuticle sheaths and contributed towards the lack of hookworms and DCs observed on the PET scaffold.

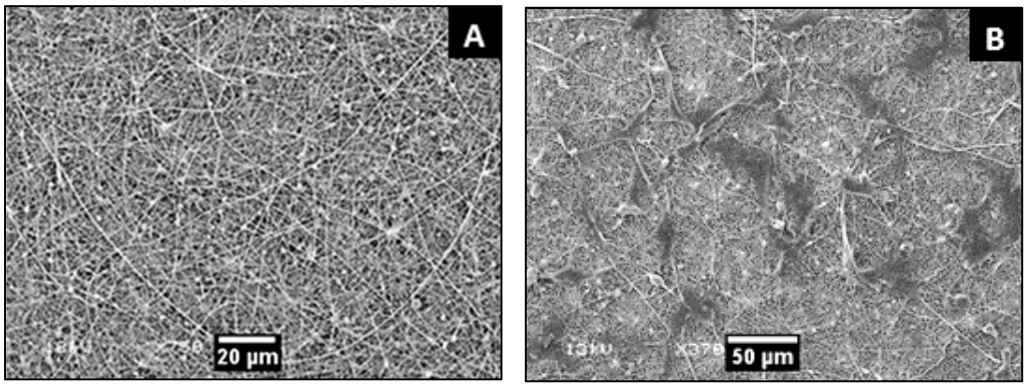


Figure 67: Scanning electron micrographs of dendritic cell interaction with *Necator americanus* larvae on PET scaffold. Untreated PET scaffold (A) and N. americanus treated scaffold (B).

Following incubation with the larvae, there are aggregates formed across the PET scaffold layer. Data shown are representative of 2 experiments.

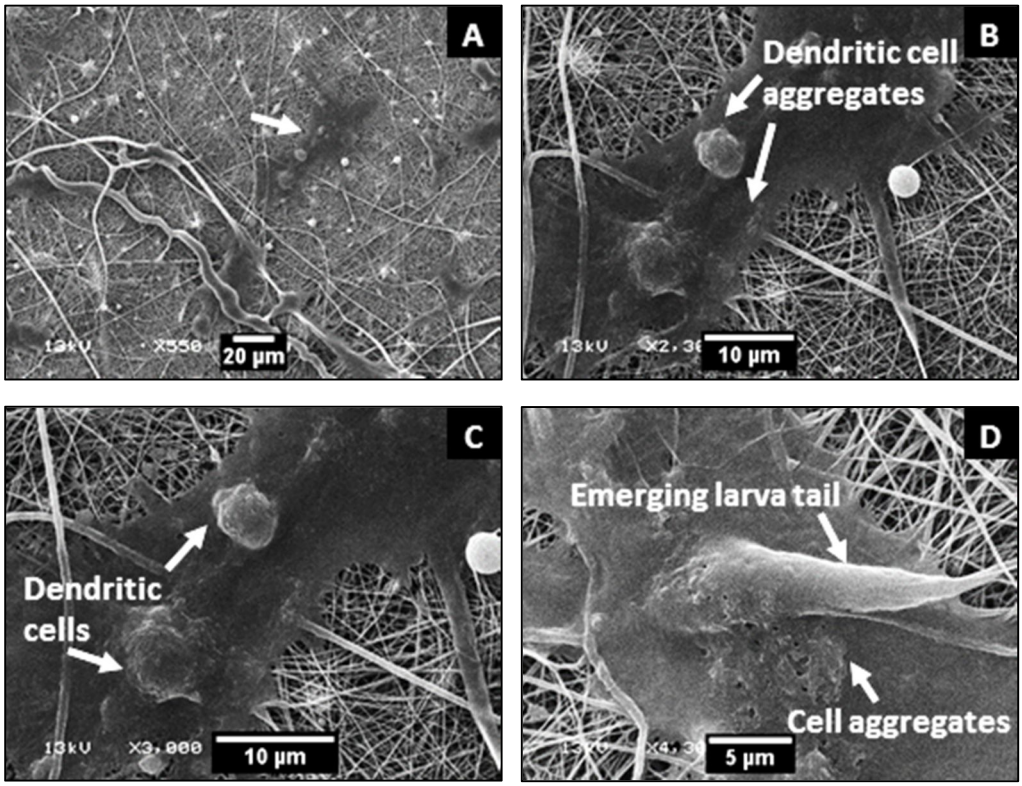


Figure 68: Scanning electron micrographs of dendritic cell interaction with *Necator americanus* larvae on PET scaffold.

Following incubation with the larvae, there are aggregates formed across the PET scaffold layer (A). Under higher magnification, the aggregates identified (B), have an ECM-like appearance. There are DCs within these aggregates, visibly sequestered/attached onto sheath-like fragments (C and D). Data shown are representative of 2 independent experiments.

Overall, the SEM data illustrated that on a porous 3D network of PET fibres, DCs and *N. americanus* larvae are capable of interacting, evident by the sequestration of DCs and aggregate formation. This rationalises and provides a foundation for studying the DC-larvae interaction in a 3D multicellular tissue-

culture on PET scaffold. The model will mimic the barrier function of the skin (HaCat), provide ECM production by fibroblasts (BJ-6) and incorporates immune cells (DCs) which act as the immune-sensing migratory component. The construct was assembled and the migration of pre-stained DCs (Hoechst) were monitored following infection with 50 pre-stained (WGA-FITC) *N. americanus* larvae for 24 hours (Figure 69).

Following confocal analysis of the scaffold layers, the localization of the larvae and its sheath were unidentified. The methodology involved in sample preparation for confocal analysis could account for the lack in fluorescent signal detected of pre-stained larvae. Following *N. americanus* stimulation, DC migration was towards the upper (HaCat) scaffold, however the majority of cells remained within the middle scaffold, with no cells detected in the lower (BJ-6) layer. In contrast, migration in the unstimulated tri-culture was primarily towards the upper (HaCat) scaffold, with some cells identified in the lower (BJ-6) layer.

Untreated	**50 *N. americanus***

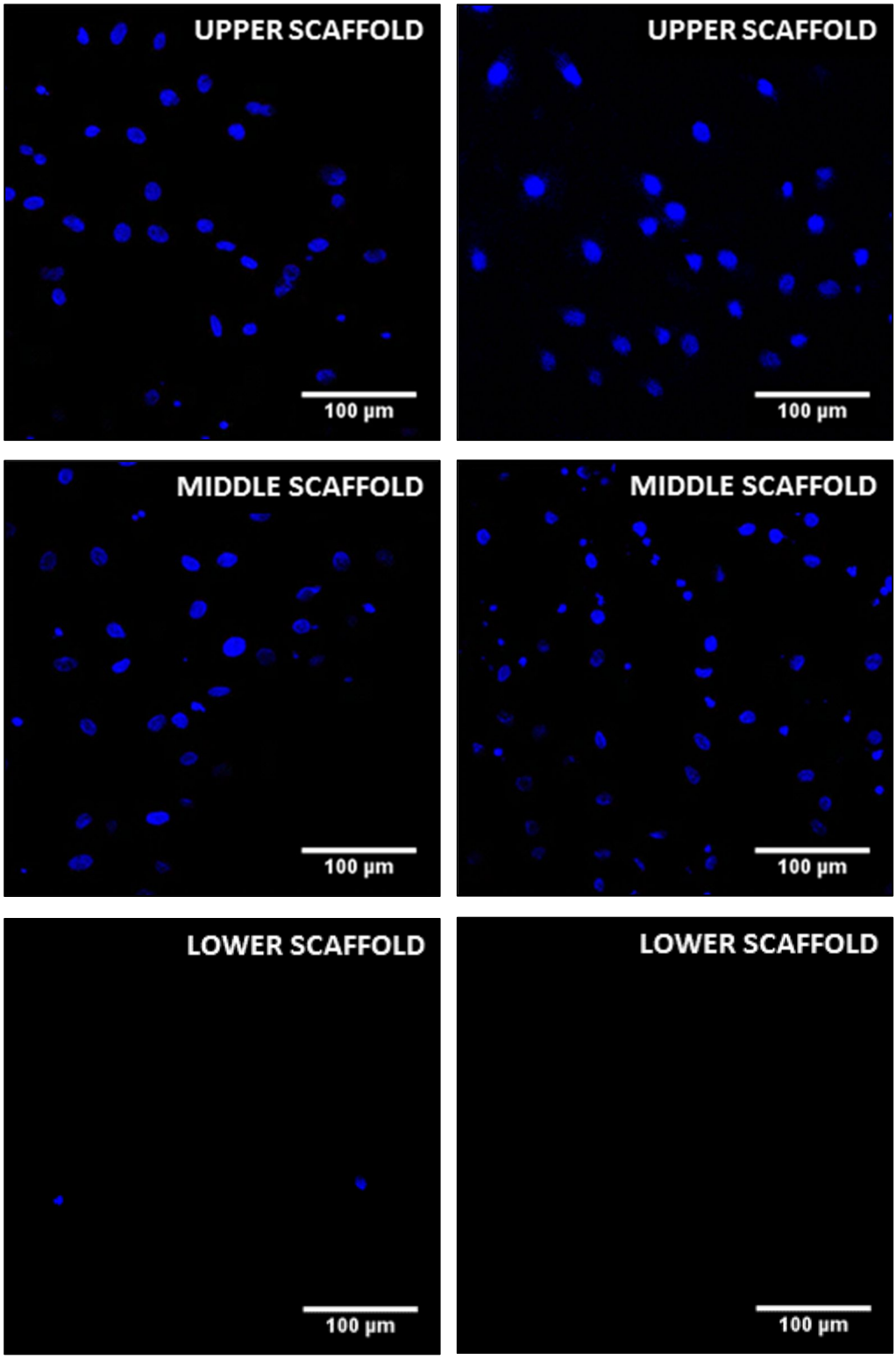


Figure 69: The migration of dendritic cells through an *in vitro* 3D tri-culture model infected with *Necator americanus* larvae.

DCs were pre-stained with Hoechst (nuclear stain). DC migration in the unstimulated tri-cultures is primarily towards the upper (HaCat) layer, with some

DCs identified in the lower (BJ-6) scaffold. Following *N. americanus* stimulation, migration was only towards the upper (HaCat) scaffold, however the majority of DCs remained within the middle scaffold. Data representative of a single experiment.

5.2 Discussion

5.2.1 The development of a functional and robust tri-culture skin model

There is a significant shortage in the availability and development of a biologically relevant, immunocompetent and cost-effective *in vitro* model of the human skin. In addition, the growing ethical pressure to abolish the use of animals for testing, further highlights the need for an alternative and physiologically applicable experimental platform (Adler, Basketter et al. 2011, Doke and Dhawale 2015, Abd, Yousef et al. 2016). The work conducted in this chapter describes the construction of an immunocompetent 3D tri-culture model, supporting the growth of numerous cell types, which are cultured at ALI on electrospun PET scaffolds (Chau, Johnson et al. 2013, Harrington, Cato et al. 2014). The tri-culture comprises of co-cultured keratinocytes and fibroblasts, interspersed with dendritic cells within a porous 3D network of PET fibres, enabling cell interaction. This model possesses adequate cellular and structural representation of the human skin (Simpson, Patel et al. 2011, Breitkreutz, Koxholt et al. 2013), providing an ideal platform for the *in vitro* study of skin sensitizers and/or mechanism of epidermal infection. Characterization was conducted through a range of imaging microscopy techniques, illustrating the

differentiation and epidermal barrier formation of HaCat cells, and essential immune responsiveness of DC migration within the tri-culture.

Anatomically, the epidermis acts as a multifunctional defence barrier, which rests on a layer of ECM of the dermis underneath. This layer of specialised ECM functions as a physical boundary between the two skin compartments, establishing the basement membrane (basal lamina) (Breitkreutz, Koxholt et al. 2013). The basement membrane, serves as an adherence structure for the epidermis, by attaching to the inner basal surface plaques of the epidermis above, via collagen fibrils and filaments of hemidesmosomes (Simpson, Patel et al. 2011, Breitkreutz, Koxholt et al. 2013). In the context of electrospun PET, structurally, the scaffold fibre network is comparable to the collagenous connective fibrils found in the basement membrane of human skin tissue (Loh and Choong 2013, Harrington, Cato et al. 2014). The interwoven fibre structure will provide functional and mechanical support to cultured HaCat/BJ-6 cells. This was evident by the development of a homogenous ECM layer on the PET scaffold and cell permeation through the fibre network (Figure 63). Furthermore, a key advantageous feature of employing PET scaffold in tissue culture, is that it can be raised to an ALI interface, enabling the differentiation and barrier development of HaCat cells (Chau, Johnson et al. 2013, Harrington, Cato et al. 2014). The data also concur with previous work within the group (Chau, Johnson et al. 2013) which illustrated analogous scaffold fibres (SLS Selectwipes™, Nottingham, UK), promoting the proliferation of keratinocytes and fibroblasts.

In particular, BJ-6 cells were a fundamental component of the tri-culture as literature has shown the presence of both keratinocytes and fibroblasts, in bi-layered construct, to be necessary for epithelial stratification (Wojtowicz, Oliveira et al. 2014). Other studies have demonstrated the importance of paracrine signalling between the two cell types as a key factor for cell organization, highlighting stromal-epithelial interdependency (Cantòn, Cole et al. 2010). Fibroblasts constitute the majority of cells in the dermis, synthesising the connective tissue fibres found within this layer and the ECM. These cells are crucial in the cellular adhesion, growth, stability and organization of the ECM, and are essential for depositing a range of structural proteins and collagen into this construct (Tracy, Minasian et al. 2016). More importantly, single culture HaCat cells fail to develop a homogenous layer of ECM, which further emphasizes the necessity of BJ-6 fibroblast inclusion in the tri-culture.

Overall, the HaCat cells populate the scaffold fibres, and in conjunction with the layer of ECM produced, provide a structure which is highly comparable to the basement membrane and anchoring collagen fibrils, that underlie all epithelial cell sheets (Alberts.B, Johnson.C et al. 2002, Breitkreutz, Koxholt et al. 2013). The micrographs in Figure 70 illustrates the basal lamina in the cornea of a chick embryo, which is morphologically analogous to the bio-engineered PET construct.

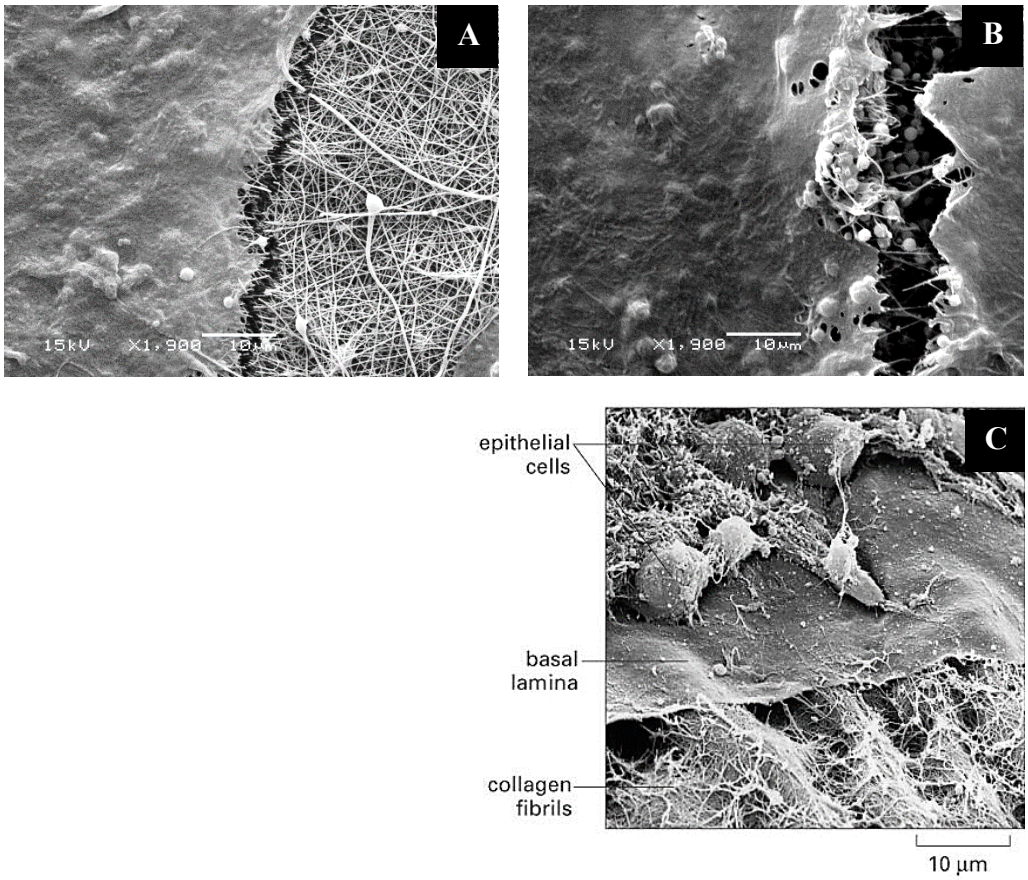


Figure 70: Scanning electron micrographs illustrating ECM layer of HaCat cells on PET scaffold at 7 (A) and 14 (B) days ALI and the polarised epithelial sheets on the basal lamina from a chick embryo.

The micrographs illustrate the highly comparable features of the homogenous ECM layer of HaCat cells (A and B) and the basal lamina of epithelial chick embryo (C). At 7 days ALI (A), the scaffold fibres are visible and adequately representative in structure to the collagen fibrils under the basal lamina (C). At 14 days ALI (B) there is a confluent layer of the ECM with HaCat cells anchored to the ECM and PET fibres. The scale bar on the images represent 10 µm. Adapted from (Alberts.B, Johnson.C et al. 2002).

Following the establishment of an *in vitro* ECM basement membrane on PET scaffold, barrier formation was analysed further. The development of tight junction protein ZO-1 and skin impedance were assessed in single and co-cultured HaCat cells. The results exhibit that single cultures of keratinocytes, cultured at ALI for a period of 14 days lacked the 3 characteristics (i.e., TEER,

ECM layer and ZO-1 expression) employed in this project to identify the development of a functional barrier. In contrast, co-cultures with dermal fibroblasts or stimulation with TGF-β (2 ng/ml) and GM-CSF (50 ng/ml) exhibited an increase in TEER and expression of ZO-1. The lack of barrier development in single culture keratinocyte was primarily due to the absence in fundamental differentiating growth factors necessary for epidermal ECM and barrier formation (Braunstein, Kaplan et al. 1994, Cho, Hong et al. 2004). This was confirmed by the abundant expression of ZO-1 and increased TEER measurements, in TGF-β and GM-CSF stimulated single culture HaCat cells.

Primarily, undisturbed keratinocytes do not express cytokines actively, with only transient levels of IL-1, 7 and TGF-β (Schroder 1995), the latter of which regulates epithelial cell growth (Ansel, Perry et al. 1990). However, cytokines such as GM-CSF (among others) are vital in the regulation of normal epidermal functions (Braunstein, Kaplan et al. 1994). Previous studies have demonstrated the essential roles these cytokines have in maintaining the functional properties of the ECM i.e., wound healing (Pakyari, Farrokhi et al. 2013), and the differentiation of epidermal keratinocytes (Braunstein, Kaplan et al. 1994). For instance, *ex vivo* mice studies have reported adverse effects following the postnatal depletion of TGF-β receptor II in dermal fibroblasts (Mauviel 2009). This was marked by an inhibition of dermal regeneration, wound healing, re-epithelialization, and differentiation. These mice also developed severe multifocal inflammatory disease, highlighting the vital role of TGF-β signalling in the skin (Mauviel 2009). Other studies have also shown GM-CSF cytokine expression during epidermal skin wounds, initiating healing in an autocrine

manner, therefore establishing a fundamental role for GM-CSF as a complex pleiotropic cytokine (Mann, Breuhahn et al. 2001).

Moreover, in the context of single HaCat cell cultures, the primary constituents of the ECM and dermis, namely the fibroblasts (BJ-6 cells), are absent (Mouw, Ou et al. 2014). This results in a deficiency of differentiating factors, provided by the supporting fibroblasts *in vitro*/*vivo* (Werner, Krieg et al. 2007, Wojtowicz, Oliveira et al. 2014), which are necessary for epidermal ECM and barrier formation.

5.2.2 The immune sensing function in a 3D skin tri-culture model

The 3D PET layers of co-cultured HaCat and BJ-6 cells were then employed to construct an immunocompetent skin model. (Werner, Krieg et al. 2007). While there are other fundamental immune cells, i.e., macrophages and mast cells (Werner, Krieg et al. 2007, Malissen, Tamoutounour et al. 2014), in terms of incorporating their immune component within the culture, the primary objective was to establish an easy to assemble model of human skin. The characterization of DCs have been extensively studied, particularly as these are comparable to LC immune responses to chemical sensitizers (in 2D cultures) (Tuschl and Kovac 2001). Additionally, DCs are readily generated, as described in the methodology (Salazar, Hall et al. 2016), and on these basis were selected as the immune sensing component of the tri-culture. Continuing, the migration of DCs

within the tri-culture was monitored as these immune cells directly depend on their ability to migrate to the site of infection and capture the invading pathogenic antigens (Martin-Fontecha, Lanzavecchia et al. 2009).

Overall, DCs maintained their ability to migrate to all layers within the tri-culture, however following LPS stimulation, migration was predominantly towards the upper scaffold. This was expected as immature DCs would primarily migrate to the upper layer following sensitization (Martin-Fontecha, Lanzavecchia et al. 2009), which also concur with previous data within the group (Chau, Johnson et al. 2013, Harrington, Cato et al. 2014). Intriguingly, although DCs were capable of migrating towards the lower layer, this was particularly increased in the presence of BJ-6 fibroblasts on the lower scaffold (model A).

The observed increase in migration was possibly due to the stromal microenvironment and DCs cross talk, as fibroblasts capacity to potentiate the migratory function of DCs has been demonstrated in an *in vitro* 3D collagen I matrix. These studies reported that activated DCs stimulate fibroblasts to secrete matrix metalloproteinase-1 and prostaglandin E2, effectively enhancing DC migration within the collagen matrix (Saalbach, Janik et al. 2015). Interestingly, other studies have illustrated immature DCs to interact via β_2 integrins with CD90 and ICAM-1 on dermal fibroblasts. These co-cultures resulted in the maturation of immune cells, confirmed by the upregulation in phenotypic markers i.e., CD83, CD80, CD86 and HLA-DR (Saalbach, Klein et al. 2007).

In the context of increased migration within the tri-culture model (A), dermal fibroblasts can potentially act as regulators of DC maturation, which could affect cutaneous immune responses and promote migration (Saalbach, Klein et al. 2007). Building on the possibility of DC maturation, the DCs observed on the middle scaffold in tri-culture model (A) appeared larger and notably clustered. Studies have shown that DCs (following maturation) are larger in size (10-15 μm) in comparison to immature DCs, estimated to be around 6-9 μm (Dumortier, van Mierlo et al. 2005). It is imperative to note that the presence of stromal-epithelial cross-talk and subsequent altered microenvironment could result in this observation. However this remains to be clarified through DC recovery from both tri-culture models and phenotypically analysing the cells for maturation markers (i.e., CD83, CD80, and CD86) (Salazar, Hall et al. 2016). Nonetheless, this could have been due to the small number of experimental repeats, as such observations have not been reported in previous literature (Chau, Johnson et al. 2013, Harrington, Cato et al. 2014).

Finally, DCs in the attempted Necatoriasis skin model remained primarily within the middle scaffold, with some migration to the upper layer. In theory, following infection, *N. americanus* larvae could possibly burrow through the upper scaffold and encounter DCs on the middle layer. This would localise the immune cells within this scaffold. Furthermore, there was no migration to the lower layers, which was probably be due to immune cell sequestration onto the larvae sheath encountered in the upper and middle scaffolds, preventing DC migration following antigen capture. A plausible mechanism whereby the exsheathed

larvae can then escape to the lower (vasculature/fibroblast) scaffold layer; however the validity of such speculation is yet to be investigated in detail.

5.2.3 Conclusion and future work

Collectively, this model is simple to assemble with a robust structure enabling the separation of its readily made, interchangeable (i.e., with other cell types), scaffold layers. The tri-culture is restricted to a 1 week time period due to DC migration, subsequent maturation and functional alterations following stimulation. Nevertheless, this permits sufficient time for sensitization and infection studies.

The future directions of the constructed tri-culture in the context of this thesis would be to optimise the methodology of post-analytical techniques and increase experimental repeats. For instance, the staining of ZO-1, pan-cytokeratin and Hoechst within the 3D layered scaffold could be improved, due to sub-optimal protocols, particularly with regards to DC migration. In addition to the high number of DCs migrating towards the upper (HaCat) scaffold, the Hoechst dye leaked out of the DCs, evident by the stained HaCat cell nuclei on the upper layer. Furthermore, as larvae secretions have been shown to disrupt vascular endothelial cadherin and F-actin (Souadkia, Brown et al. 2010), it is imperative to assess the effect of live ensheathed *N. americanus* larvae on ECM and ZO-1 expression in HaCat cells.

It would also be better suited to identify larvae and sheath localisation within the tri-culture, through fixation in 4% PAF and subsequent immersion in molten paraffin wax (Chau, Johnson et al. 2013); preparing the construct for histochemistry analysis (Hewitson, Wigg et al. 2010). The dehydration of the sample and haematoxylin/eosin staining steps would not be necessary (removing further possibility of larvae washing away) as the *N. americanus* larvae are relatively large in size (300-400 μm) (Pasuralertsakul and Ngrenngarmlert 2006). The primary objective in these experiments would be to section the wax embedded samples and identify (via light microscopy) which layer of the scaffolds the larvae/sheath have localised on.

In conclusion, there is a significant lack in the availability of validated *in-vitro* platforms which enable predictability of hypersensitivity reactions towards drugs in the pre-clinical phase. The platform described in this chapter provides a basic model for skin sensitization, which can be further developed and optimized to screen the allergenic potential of drugs (Galbiati, Papale et al. 2016). Albeit the skin tri-culture presented requires further optimization, it provides an instrumental tool for academic research, a platform for basic innate immune responses, and can assist in the alternative use of employing *in-vitro* models as opposed to animal testing.

CHAPTER 6: General discussion and future work

6.1 General discussion

Necatoriasis is a neglected tropical disease caused by the parasite *N. americanus* which infects and reinfects approximately 500 million individuals worldwide, following which no lasting immunological memory develops in the host (Loukas, Hotez et al. 2016). Therefore the primary objective of this thesis was to elucidate the unknown interactions between the infective larval stages (L_3) of *N. americanus*, with key components of the human innate immune system.

The work described in Chapter 3, provides novel insights into early immunological events at the interface of DCs and *N. americanus* larvae, and could explain how L_3 evade immunity upon initial interaction with antigen presenting cells. For the first time, the immune evasive role and benefit for the *N. americanus* to maintain its cuticle sheath from the non-infectious L_2 stage, was illustrated. Upon the encounter of the ensheathed larvae, immature DCs are sequestered onto the cuticle sheath, via a mandatory interaction with both DC-SIGN and MR, triggering larval exsheathing. Intriguingly, DCs continue to bind and form dense cell aggregates around the discarded sheaths, exhibiting negligible interactions with the exposed larvae. The difference in distinct chemical signatures, between the sheath and larvae surface, is in theory highly beneficial. This would enable parasite migration and escape into the vasculature without being recognised or challenged by APC, through the sheaths antigenic diversion. Therefore, based on these findings, the biological relevance of the

heavily glycosylated larvae sheath (Kumar and Pritchard 1992) appears to be tailored for bypassing innate immune cells at the initial site of infection. Nevertheless, although the data clearly suggests that the larvae are actively targeting DC-SIGN and MR to sequester DCs onto the larvae sheath; the exact mechanisms of interactions remain to be established.

The APC function of DCs, depends on their ability to migrate to the site of infection. This function is indispensable for the maintenance of immune surveillance and tissue homeostasis, initiating protective tolerogenic and pro-inflammatory responses (Imai, Minamiya et al. 2012). Rationally, the sequestration of immune cells could be a plausible explanation for the poor T cell responses observed in human hookworm infection. This would hypothetically prevent the necessary migration of DCs to lymph nodes, in order to stimulate an effective adaptive immune response (Martin-Fontecha, Lanzavecchia et al. 2009).

Furthermore, DCs incubated with viable axenised larvae exhibited an immature phenotype as evidenced by the low expression of the maturation markers CD80, CD83, CD86, CD40, and HLA-DR. Nevertheless, DCs remain responsive to LPS in co-stimulations with the larvae, which was perhaps due to the axenised state of the hookworms. Arguably, there was a specific down regulation in MR expression, which has also been identified as a key factor in DC sequestration. In addition, the data presented in this chapter concur with and expand on

previous literature in respect to parasite impaired DCs. The study reported monocyte derived DCs from infected volunteers (as opposed to non-infected), to maintain an immature phenotype following LPS stimulation (Fujiwara, Cancado et al. 2009). Additionally, while the larvae on its own do not induce any cytokine production by DCs, they seem to suppress LPS induced cytokine production. Overall, this data further supports the theory of *N. americanus* immune suppressive properties (Loukas and Prociv 2001).

Finally, the work conducted in Chapter 3 identified that albeit LPS-conditioned media had no effect on the larvae; mature DCs have the capability to lyse and render the infective L_3 *N. americanus* non-viable. This observation highlighted a possible modified paracrine signalling response of mature immune cells in culture with the parasite.

Continuing, the research presented in Chapter 4 investigated the physical interactions between the *N. americanus* larvae and human epidermal keratinocytes (HaCat). The data alluded to a significant preference of larvae maintaining their sheath during the initial stages of infection, which in theory is highly beneficial. The larva sheath, as illustrated in Chapter 3, is a fundamental feature which enables DC sequestration and immune evasion. Furthermore, larvae were also notably slower to discard their sheath in culture with HaCat cells and were in a lethargic state as observed in DC cultures. These findings correspond with a period of larvae dormancy observed in the skin stages of

infected animal models (Behnke, Paul et al. 1986). Previous studies have shown that the *N. americanus* larvae are well adapted and employ behavioural strategies, which promote host invasion (Haas, Haberl et al. 2004). Rationally, the observed period of inactivity in conjunction with the cuticle sheath, could be another hookworm strategy. Following skin penetration, ensheathed dormant larvae are possibly establishing a site of immune privilege, by enabling time for DC to migrate to the site of infection. This would consequently ensure the sequestration of DCs onto the larval sheath, followed by exsheathing and escape into the vasculature. Nonetheless, the validity of such speculation is yet to be investigated using appropriate *in vivo* models.

The larvae are 300-400 μm in size, and the access gained to the vasculature was thought to be mediated through an increased permeability of the skin barrier as shown in endothelial cells (Souadkia, Brown et al. 2010). However, despite the deterioration in morphology of *N. americanus* infected HaCat cells, IL-8 levels did not increase. It would therefore seem likely that mechanical burrowing action in addition to secreted skin proteases enable the larvae to penetrate the epidermal layers of the skin. Furthermore, the cellular destruction observed also concurs with early *in vitro* studies in human skin (Matthews 1982, Brown, Girod et al. 1999), providing more proof that larvae depend on secreted proteases to digest components of the skin.

Chapter 5 in this thesis describes the construction of an immunocompetent tri-culture model of the human skin. This model encompasses co-cultured HaCat and BJ-6 cells, interspersed with DCs within a porous 3D network of PET fibres at ALI (Chau, Johnson et al. 2013, Harrington, Cato et al. 2014). The PET tri-culture model illustrated the resemblance and structural representation of the human skin basement membrane and epithelial barrier function (Alberts.B, Johnson.C et al. 2002, Simpson, Patel et al. 2011, Breitkreutz, Koxholt et al. 2013). The DCs incorporated an immune sensing function, by migrating through the 3D network of PET fibres. However, due to DCs phenotype and function modifications (Ueno, Klechevsky et al. 2007), the tri-culture is limited to a 1 week time period. Overall, this model is an instrumental tool for academic research, highlighted by a robust platform feature, with easy to assemble components and interchangeable scaffold layers.

6.2 Future work

The limited source of *N. americanus* larvae availability for research (Tanasescu and Constantinescu 2015) prevented further investigations into key observations derived from the data presented in this thesis.

For instance, the co-culture of LPS matured DCs with ensheathed *N. americanus* larvae resulted in the lysis of the parasite, alluding to an altered paracrine signalling response. It is paramount to identify the specific, mature DC

cytokine/chemokine secretions in the microenvironment, as this could possibly be utilised in vaccines targeting the early stages of infection. Additionally, investigating the effects of mature DC culture media (following incubation with the larvae) on live ensheathed *N. americanus* is vital. This would identify whether hookworm lysing is predominantly due to a secreted factor or co-dependant on the presence of mature immune cells.

Furthermore, the data presented also provides a strong rationale for targeting specific lectins on larvae surface and/or silencing MR and DC-SIGN expression on DCs, in order to fully elucidate the cross-talk between the sugar moieties on the larvae sheath and the CLRs on DCs. Finally a microarray comparison of known keratinocyte and DCs ligands on the larvae sheath, would highlight possible receptor-ligand interactions.

More importantly, the *N. americanus* larvae employed for research are ensheathed, highlighting a possible differential interaction between immature DCs and exsheathed larvae. This is an imperative area for research as it could elucidate whether immature DCs are capable of interacting with the exposed larva, in the absence of the L_2 cuticle sheath.

The tri-culture provides a proof of concept for *N. americanus* infection, which is paramount in elucidating the behaviour of larvae in a 3D tri-culture environment. However his requires further optimizations, due to suboptimal

protocols of post-analytical techniques. Nonetheless, it is vital tool for the observation of larvae and sheath localisation within an *in vitro* model of the skin. Subsequently, the cytokine microenvironment plays a key role in DC interaction with *N. americanus*, and thus should be analysed in addition to assessing the phenotype and function of all cell types present.

Conclusively, it is imperative to point out the axenised state of the larvae employed for research in this thesis. Therefore to provide a better immunological understanding between the larvae and components of the innate immune system, it is fundamental to employ un-sterile hookworms for all further research.

Collectively the novel findings presented and recommendations made throughout this thesis are exciting areas for further research, which will indefinitely aid in the continuous battle against human *N. americanus* infections.

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