



The development of a stratified keratinocyte model for *Chlamydia trachomatis* pathogenesis studies

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Declaration

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DEDICATION

This dissertation was written in the memory of

Shamduth Jadoo

Bahadur Rambaran

Tamara Ann Gabriel

and

Ashish Singh

Your impact on my life remains unparalleled.

*“Every life comes to an end when time demands it. The loss of life is to be mourned – but only if the
life was wasted”*

- Leonard Nimoy as Spock

“Star Trek: The Animated Series”: Yesteryear, ep. 1973

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LIST OF ABBREVIATION AND ACRONYMS

μm Micrometres γ -irradiation

Gamma irradiation

2D Two Dimensional

BPE	Bovine Pituitary Extract
C. muridarum	Chlamydia muridarum
CGM-P	Chlamydia Growth Medium for Propagation
Conc.	Concentration
CO ₂	Carbon Dioxide
C. trachomatis	Chlamydia trachomatis
Da	Dalton
DAPI	4'6-diamidino-2-phenylindole
DEDs	Dermal Equivalent Substrates
DMEM	Dulbecco's Modified Eagles Medium
DNA	Dioxyribonucleic Acid
EB	Elementary Body/ies
EBSS	Earle's Balanced Salt Solution
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetra-acetic acid
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
Fe	Iron
FGF-7	Fibroblast Growth Factor 7
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor

H&E	Hematoxylin and Eosin
HBSS	Hanks Buffered Saline Solution
HEPES	Hydroxyethyl Piperazineethanesulfonic acid
IL1	Interleukin 1
IL1- α	Interleukin 1 alpha
IL1- β	Interleukin 1 Beta
InC	Inclusion Membrane Proteins
KDa	Kilo Dalton
KGF	Keratinocyte Growth Factor
KGM	Keratinocyte Growth Medium
L-Glutamine	Levo-Glutamine
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
MIF	Microimmunofluorescence
MOMP	Major Outer Membrane Protein
MOI	Multiplicity Of Infection
mRNA	Messenger Ribonucleic Acid
MTOC	Microtubule Organising Centre
N-linked	Nitrogen-linked
NaOH	Sodium Hydroxide
NEAA	Non Essential Amino Acids

NEKs/NHEKs	Normal (Human) Epidermal Keratinocytes
nm	Nanometres
PBS	Phosphate Buffered Saline
PG	Peptidoglycan
PTFE	Polytetrafluoroethylene RB
Reticulate Body/ies rhGF	Human Recombination
Epidermal Growth Factor RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic acid SPG
Sucrose Phosphate Buffer	
TGF	Transforming Growth Factor
TGF- α	Transforming Growth Factor alpha
TPA	Tissue Plasminogen Activator
TTSS	Type III secretion system
UKZN	University of Kwa-Zulu Natal
WHO	World Health Organization
w/v	Weight per volume

ABSTRACT

A number of different methods to generate stratified keratinocyte layers have been published. These involved the use of normal human epidermal keratinocytes (NHEKs/NEKS), which have a better ability to stratify compared to HaCaT keratinocytes, which usually require supplemented growth factors or stromal interactions with fibroblasts to do so.

This study aimed to generate a model of stratified keratinocytes, closely resembling *in vivo* skin, using HaCaT cells and to demonstrate the effect that *C. trachomatis* has on these layered keratinocytes, allowing us to gain insight on the pathophysiology of this organism.

All cells and bacteria were propagated and titrated according to conventional protocols. HaCaT cells were subcultured upon confluence, seeded (1×10^6 cells/ml) onto collagen-coated PTFE Transwell membrane inserts and incubated at 33°C and 37°C for 24 days to allow differentiation and stratification. Once cells became confluent they were exposed to the air-liquid interface and fed with KGM Gold (Lonza) supplemented with 10% FBS and additional calcium. Thereafter, cells were fixed in 3.7% phosphate-buffered formaldehyde, embedded in a paraffin block, sectioned, stained and viewed. Hematoxylin and Eosin (H&E) staining was used to determine the resemblance to *in vivo* human skin. Immunofluorescence was used to detect keratin 10, keratin 14 and involucrin which are markers of keratinocyte differentiation. Stratified keratinocyte layers were infected with *C. trachomatis* and this was confirmed using the MicroTrak® *C. trachomatis* Culture Confirmation Test Kit. Subsequent changes to the layers were also observed and recorded.

It was shown that HaCaT cells grown at the air-liquid interface on collagen-coated PTFE Transwell membrane inserts were able to stratify at 33°C. However, more layers of keratinocytes were seen at 37°C after the same duration of incubation (24 days). Keratin 10, keratin 14 and involucrin were all detected in the layers grown at both temperatures, suggesting that the keratinocytes had committed to differentiation. However, the fluorescence seen at 33°C for keratin 10 and involucrin was more intense as compared to that seen at 37°. This suggests that although stratification was faster at 37°C, differentiation was quicker at 33°C.

C. trachomatis was able to infect layered keratinocytes grown at both temperatures although not all layers formed at 33°C were infected. Degradation of keratinocyte layers after infection with *C. trachomatis* was more prominent in those grown at 37°C, which is in keeping with previous findings that the optimum growth temperature of the *C. trachomatis* LGV biovar is 37°C.

This study provided a novel insight in suggesting the manner in which *C. trachomatis* is able to infect and migrate through *in vivo* skin, leaving room for further studies in which similar methods of generating stratified keratinocytes may be used to better understand the pathophysiology of various other organisms that affect keratinocytes.

CHAPTER 1 – INTRODCUTION

Chlamydia trachomatis is a gram negative, sexually transmitted pathogen with a characteristic obligate intracellular life cycle, essential for survival (Caldwell *et al.* 1981).

Chlamydiae predominantly infects columnar epithelial cells, causing an inflammatory response which further orchestrates both, an innate and adaptive immune response (O'Connell *et al.* 2006).

C.trachomatis is associated with a number of various diseases (Bobo *et al.* 1997). In 1995, the World Health Organization (WHO) reported 89 million new findings of genital Chlamydia infections, indicative of the effect that chlamydial infections have on a global scale, and the economic strain it causes (Schachter, 1999). More specifically, the lymphogranuloma venereum (LGV) serovars L1, L2 and L3 cause a more invasive sexually transmitted disease called Lymphogranuloma venereum (Schachter, 1999). This invasive bacterium travels down the lymphatic channels after passing through the epithelial cell layer. Thereafter, multiplication occurs within mononuclear phagocytes of the lymph nodes (Belland *et al.*, 2004). Invasion of the lymphatic tissue ultimately results in severe clinical symptoms such as abscesses, lymphatic fibrosis, elephantiasis of genitalia, and anal strictures (Mabey and Peeling, 2002).

Cases of LGV infections are common in Africa, the Caribbean and parts of Asia (Perine and Stamm, 1999). In 2003, more modern and revolutionized countries suffered an epidemic of LGV in homosexual males, where outbreaks of LGV proctitis were commonly reported in the Netherlands and Western Europe, despite the misconception of being relatively rare in modern times. (Nieuwhuis *et al.*, 2004).

Previous studies that investigated the *in vitro* infection of *C.trachomatis* in keratinocytes were only conducted on monolayers of human keratinocytes, which does not constitute part of the human *in vivo* environment. Therefore, these studies did not give a clear depiction of the pathogenesis of the organism.

This study aims to generate a stratified keratinocyte model *in vitro*, which closely resembles the *in vivo* human epidermis, using tissue-culture techniques. The model will then be infected with the *C.trachomatis* L2 reference strain, in an effort to assess the ability of *C. trachomatis* to migrate through the multi-layered human keratinocyte structure. Overall, the development of these keratinocyte infection models, *in vitro*, allows for a clearer understanding of the pathogenesis of *Chlamydia trachomatis*, essentially opening room for improving treatment of infected patients.

This study was approved by the Research Ethics Committee of the Nelson R Mandela School of Medicine, University of KwaZulu Natal. Reference number: BE021/16 (sub-study of BE220/13).

CHAPTER 2 – LITERATURE REVIEW

2.1 Chlamydia trachomatis

Chlamydia trachomatis is a Gram negative bacterial pathogen which causes both genital tract and eye infections in humans. It comprises of 15 serovars (A, B, Ba, C-K and L1-L3), grouped based on antigenic differences in the chlamydial major outer membrane protein (MOMP) (Caldwell *et al.* 2003; Schachter, 1999; Mabey *et al.* 1999).

The serovars are further grouped into biovars which differ in pathophysiology. Serovars A-C make up the trachoma biovar (Mabey *et al.* 1999), serovars D-K the urogenital biovar and L1-L3 the lymphogranuloma venereum (LGV) biovar (Moulder, 1991). The trachoma biovar consists of serovars A, B, Ba and C, which cause trachoma whereby the urogenital serovars D-K cause urethritis in male and urethritis and cervicitis in female (Carlson *et al.* 2004). Both, trachoma and urogenital strains have the ability to infect epithelial cells of the conjunctiva and genital tract respectively, thereby causing disease that is specific to the particular organ system (Miyairi *et al.* 2006). The range of diseases caused by the trachoma biovar is limited to chronic conjunctival infection, whereas, urogenital strains cause a wider range of both acute and chronic genital tract infections but also a form of active conjunctivitis (Leonard & Borel, 2014). Chlamydia of the LGV biovar are invasive (Schachter & Moncada, 2005) in the sense that these infect tissue underlying the primary target of epithelial cells. The other chlamydial biovars are non-invasive.

Interestingly, the *C. trachomatis* serovars have approximately 99.6% of shared DNA, even though there is a substantial variation in their respective disease spectrum, indicating that variations are as a result of a minute number of genetic differences (Carlson *et al.* 2004).

C. trachomatis is transmitted through infected body secretions and infects mostly mucosal membranes namely; the cervix, rectum, urethra, throat and conjunctiva. The most common method of transmission of serovars D-K and L1-L3 is through sexual contact (Wang, 1999).

2.2 Lymphogranuloma venereum (LGV)

LGV is an infection of the skin and lymphatic system caused by *C. trachomatis* of the LGV biovar (Schachter, 1983). The infection develops when the bacterium gets in contact with viable epithelial cells in the deeper layers of the skin. This results in the formation of a genital ulcer. From there, the bacteria pass through the epithelial cell layer and travel along the lymphatic channels towards the inguinal lymph nodes where it multiplies within mononuclear phagocytes causing inguinal lymphadenitis, the secondary stage of infection. If left untreated, a tertiary stage may follow which involves the occurrence of fibrosis of the lymphatics, resulting in varying degrees of lymphatic obstruction, chronic oedema and strictures. These symptoms are usually irreversible (Ward *et al.* 2007).

2.3 Biology

2.3.1 Structure and morphology

C. trachomatis is a gram negative obligate intracellular bacterium, which appears in two different forms (Ward 1983). The elementary body (EB) is the infectious, metabolically inactive form while the reticulate body (RB) is non-infectious, metabolically active and replicates (Ward 2002).

The EB is small and compact with a ridged cell wall that provides osmotic resistance, thus rendering it more resilient during the transfer from one host cell to another (Ward 1983). It is spherical in shape with a diameter of approximately 350 nm (Ward 2002) and comprises of cytoplasm and compactly-packed DNA together with 3 binding-proteins, which are H1like proteins (Wagar & Stephens 1988). In human cells these H1 proteins maintain the overall structure of the chromatin network. Two of the H1-like proteins in chlamydia (Hc1 and Hc2) are involved in condensation of the DNA when EB are formed. The activity of Hc1 is controlled by regulatory RNA (IntA) which prevents the transformation of RBs into EBs (Grieshaber *et al.* 2006).

The RB is much larger than the EB (Bavoil *et al.* 1984). It is pleomorphic in shape with a diameter of approximately 1µm (Ward 2002). Being metabolically active, RBs lack the chlamydial histone analogues that condense the nucleoid in EBs (Hacktadt *et al.* 1991). The diffuse nucleoid of RBs, allows transcription and synthesis of proteins (Ward 2002). Since *C. trachomatis* is a gram negative organism, both the EB and RB are surrounded by a cytoplasmic membrane and a layered cell wall (Ward, 1983). In a study by Matsumoto & Manire (1970), the cell walls of both EBs and RBs were examined and compared. It was found that the cell wall of the EB had a high phospholipid and amino-acid content, as expected in typical gram-negative organisms. However, the cell wall of the RB had a decreased amount of phospholipid, with no detectable amino-acids. Manire (1966) demonstrated that hexagonal subunits are synthesized during the maturation of RB to EB which form part of the interior of the cell wall. The chemical composition thereof remains unknown. Manire (1966) also elucidated that the synthesis of the subunits is prevented by penicillin and stated that the primary purpose of the subunits is to provide the cell wall of the EB with strength and rigidity, which is absent in the RB, making it more fragile in comparison. This layer of subunits is known as the P-layer (Matsumoto & Manire, 1970). The transformation from EB to RB is dependent on bacterial protein synthesis (Gerard *et al.*, 1997). There is expression of chlamydial 16S rRNA during the transformation of EB to RB, making it a marker for both, replicating and viable non-replicating organisms (Mathews *et al.*, 1999). Since rRNA constitutes part of the chlamydial ribosome, it is essential for function of the organism (Engel *et al.* 1987). Using reverse transcription-polymerase chain reaction assays, Gerard *et al.* 1997 tracked chlamydial RNA and discovered that transcripts from proximal rRNA promoters are seen 4 hours after the EB binds to the host cell and distal rRNA promoters were detected 6 hours after infection, indicating that protein synthesis occurs at a very early stage during the transformation of EB to RB.

Like other Gram negative bacteria, the cell wall of *C. trachomatis* consists of a cytoplasmic membrane, a peptidoglycan (PG)-like layer and an outer membrane. However, the cell wall of this organism has a few unique structural features, namely no detectable muramic acid, atypical lipopolysaccharide (LPS) and a specific outer membrane protein (MOMP) (Liechi *et al.* 2014).

Muramic acid, an amino sugar acid, is a component of peptidoglycan adding to the structural component of most bacterial cell walls (Liechi *et al.* 2014). It has been established that chlamydia has genes coding for PG and that they are susceptible to beta-lactam antibiotics that target PG, even though PG as such is not detected in chlamydial species.

This is known as the “chlamydia anomaly” (Ghuysen & Goffin 1999). Earlier studies elucidated the detection of muramic acid in small quantities in *C.trachomatis* from embryonated eggs (Jenkin, 1960). However, these studies made use of colorimetric methods which is not a reliable means of differentiating between smaller amounts of chemically similar compounds in complex mixtures. (Fox *et al*, 1990). In such cases, glucosamine and various amino sugars interfere in the colorimetric detection of muramic acid, which is common when muramic acid is found in small amounts (Fox *et al*, 1990). Furthermore, Fox *et al*, 1990, suggested that trace levels of muramic acid is not sufficient to synthesize a peptidoglycan, implying that the exact mechanism of action of antibiotics such as penicillin against chlamydiae remains unclear.

Chlamydial LPS makes up part of the outer membrane of chlamydia. It differs from other gram-negative bacteria in that it has two of the three antigenic domains usually found in LPS (Weisburg *et al.* 1986). The cell wall is selectively permeable, thus protecting the EB whilst it is outside the host cell (Ghuysen & Goffin. 1999) and maintains a fixed shape and resilience to the stress of osmosis. Instead of muramic acid, sulphur bridges between cysteine-rich proteins allow for the rigid structure and ability to withstand osmotic pressure (Bavoil *et al.* 1984).

Chlamydia have a genus-specific LPS as part of the outer membrane (Brade *et al.* 1985). The key difference between chlamydial LPS and that of typical gram negative bacteria lies in the lipid-A moiety, comprising of 5 fatty acyl groups instead of the usual 6. The fatty acyl groups are long and comprise of normal fatty acids rather than hydroxyl fatty acids. This results in reduced endotoxicity of chlamydial LPS relative to that of typical bacteria (Heine *et al.* 2003). This allows *chlamydia* to develop asymptomatic cervicitis for which no treatment is sought. From there chlamydia travel up the genital tract to the sterile oviducts causing infertility (Ingalls *et al.* 1995). These asymptomatic infections also allow for effective transmission.

The outer membrane contains the major outer membrane protein (MOMP), which offers heterogeneity in 4 segments, each having different antigenic characteristics. This allows for the characterization of the strains into various serovars that can be identified by microimmunofluorescence (MIF) tests. In most specialised chlamydia laboratories, MIF is replaced by sequencing of the genes coding for the antigens (Stothard *et al.* 1998). MOMP is a glycosylated cysteine-rich protein with a molecular mass of 40 000Da (Newhall and Jones, 1983). MOMP is situated on the outer surface of the infectious EB, making it essential for the interaction between *C.trachomatis* and the mammalian host cell since the glycan moieties of MOMP is largely responsible for cell adhesion (Su *et al.*, 1990). Other imperative functions of MOMP include the maintenance of the overall structure of the outer membrane, assisting in the formation of pores (Ortiz *et al.*, 1996) and the regulation of nutrient and metabolite exchange for the RB (Bavoil *et al.*, 1984). In addition, Ortiz *et al.*, 1996, demonstrated that human T-cells are activated by peptide epitopes that are found within MOMP, resulting in cell-mediated immunity.

2.3.2 Life Cycle and Development

Chlamydia are obligate intracellular pathogens (Moulder, 1991). They replicate intracellularly within a membrane-bound structure called an “inclusion”, the membrane of

this inclusion, which originates as host cytoplasmic membrane, is altered by the insertion of chlamydial inclusion membrane proteins or Inc (Mital *et al*, 2010). These Incs play a role in the evasion of lysosomal fusion and degradation.

The life cycle of chlamydia involves the adhesion and entrance of an infectious EB into a target cell (Elwell *et al*, 2016). Following entrance into the cell, the membrane bound chlamydia move along microtubules to the microtubule organising centre (MTOC). This movement is dynein-dependent but needs additional chlamydial proteins to move to the perinuclear region. Within 6-9 hours of entry, the EB swells (as the DNA becomes more diffuse) and ribosomes are synthesised, followed by the formation of the RB (Moulder, 1991).

Upon *in vitro* infection of cells with chlamydia, Yasir *et al*. 2011, noticed a substantial increase in the markers of autophagy. This is the intra-cellular degradation of nonfunctional organelles and is therefore a mechanism that can be used by the cell to remove chlamydia inclusions. Yasir *et al*. 2011 found that this defensive autophagy mechanism is activated if the chlamydial species is not adapted to the host, e.g *C. muridarum* in human cells. However, this defence mechanism was down regulated when the human parasite *C. trachomatis*, L2 was used.

The RB body divides by binary fission, with a generation time of approximately 2-3 hours whilst the inclusion simultaneously intercepts sphingomyelin and cholesterol containing vesicles from the exocytic pathway with a preference for sphingomyelin (Hackstadt *et al*. 1995, Moore et al, 2008). The subsequent reticulate bodies are reorganized into elementary bodies. Thereafter, these elementary bodies are released to infect other target cells (Moulder, 1991).

The exit of *C. trachomatis* from host cells may take place by one of two methods: cell lysis or extrusion. The difference between the two is that the host cell dies during cell lysis while it survives during extrusion. Chlamydia induced cell lysis is a quick process facilitated by proteases (Hybiske & Stephens, 2007). The inclusion membrane ruptures and the cellular

organelles and cell membrane become permeable and lyse. In contrast, extrusion involves the exit of EBs from the host cell in an organised manner. Firstly, part of the membrane protrudes from the infected cell, resulting in the inclusion being snipped off into different compartments and substances attaching to the cell periphery. Thereafter (~2-3 hours), all chlamydial particles bound to the membrane are released. This process is advantageous to the bacteria since all particles remain protected from the extracellular environment for a short amount of time and are thereby protected from the host's immune system (Hybiske & Stephens, 2007).

Wyrick, 2000 made mention that mature chlamydial inclusions move along the exocytic/extrusive pathway to the surface of the epithelium to fuse with the plasma membrane. This happens at both, the apical membrane, allowing the release of chlamydia onto the epithelial surface and the basal membrane releasing chlamydia that disseminate within the tissue. In addition, leftover osmotically unstable RB are lysed in the extracellular milieu allowing only infectious EB to enter the infection cycle

Under certain conditions, *Chlamydia* can establish a persistent growth state, where cell division comes to a halt, resulting in cells becoming morphologically aberrant and capable of returning to a normal growth state after favourable host cell conditions are restored

(Hackstadt *et al.* 1997). This phenomenon is known as “persistence” and takes place when there is a lack of nutrients and immunological attack (Wyrick, 2000). Only viable, noninfectious organisms (RBs) can become persistent whilst appearing enlarged and disintegrated during this state (Wyrick, 2000)

2.4 Effect on the host cell

As a typical feature of pathogenic gram negative bacteria, *C. trachomatis* uses a type III/contact-dependent secretion system (TTSS). The exact mechanism of this system remains unknown however, it is known that it plays an imperative role in the translocation of various

chlamydial antigens like MOMP, LPS and IncA. The system may also be involved in attaching to various hosts via injection of substances through host cells to mobilize the cytoskeleton thus orchestrating the intake of the bacterium (Wyrick, 2000).

The inclusion membrane of *C. trachomatis* serves as the connection between the bacterium and the host. It disrupts the exocytic pathway, where sphingomyelin is integrated into the inclusion membrane, after being transferred from the trans-golgi thus becoming a component of the chlamydial cell wall (Hackstadt *et al.* 1995). Similarly, acquisition of cholesterol and phospholipids takes place. Changes to the inclusion membrane are made by Incs, which are inclusion proteins that have a hydrophobic domain that constitutes 40 amino acids.

Surprisingly, there is no sequence homology to the hydrophobic domain found in the domain (Wylie *et al.* 1997; Bannantine *et al.* 2000).

Field & Hackstadt, 2002 elucidated 5 InC proteins in the membrane of *C. trachomatis* inclusions, resulting in interactions with proteins of the host that allow for signal transduction.

Interestingly, Fields & Hackstadt 2000 also found that inclusion membrane proteins that lack a hydrophobic domain also constitute part of InC proteins such as, CopN and CapL.

Heizen & Hackstadt 1997 determined that the inclusion membrane remains impermeable to molecules sized 520D to 500kD however, Grieshaber *et al.* 2002 showed that the inclusion membrane may allow for the acquisition of cytoplasmic ions.

It is known that iron is imperative for the growth of Chlamydia (Grieshaber *et al.* 2002) however, the means of acquisition of iron remain unknown. Nunez *et al.* 1990 have stated that endosomes, located close to the inclusion, contain the transferrin-transferrin receptor complexes and release Fe²⁺ after acidification and subsequent reduction. Given that the membrane is permeable by ions (Grieshaber *et al.* 2002), it is hypothesized that Fe²⁺ is transferred to the inclusion membrane via endosomes. Thereafter, they are released into the cytoplasm, allowing entrance into the Chlamydial inclusion via diffusion (Nunez *et al.* 1990). Once there is a substantial amount of organisms within the inclusion, the bacterium starts to

compete with the host for nutrients thus disrupting host cell replication (Horoschak & Moulder, 1978).

A cytotoxin in the EB of serovar D shares a substantial degree of homology with cytotoxins (LCT) A and B of *Clostridium difficile* (Belland *et al.* 2001). This cytotoxin interrupts the actin cytoskeleton and the intracellular interactions. Carlson *et al.* 2004 demonstrated that cells infected with a serovar D at an increased MOI caused the flattening of cells followed by actin depolymerisation. This was unlike cells infected with serovar L2, which maintain a constant morphology throughout.

Cytotoxin CT166 displays a DXD motif imperative for the activity of enzymes, in both bacterial and mammalian type-A glycosyltransferases, expresses glycosyltransferase activity (Belland *et al.* 2001). In a study by Bothe *et al.* 2015, HeLa cells that express the cytotoxin, were seen to also express the rounding of the cells, preventing the activity of Rho-GTPases Rac/Cdc42. Overall, this showed that CT166 has an imperative role in cellular functions, making it essential for the intracellular survival of chlamydia.

In addition, Belland *et al.* 2001, showed that the cytotoxin present in the EB is transferred to the host cell upon infection and has a virulent role, thus adding to the pathogenesis of the organism. Interestingly, Thalman *et al.* 2010 showed that the chlamydial uptake was disrupted in cells that over-expressed CT166, indicating that CT166's role as an effector-protein during the infection of host cells may vary.

2.5 Keratinocytes

Human skin is a multi-layered epithelium with self-renewal and tissue repair capabilities (Figure 2.1). The epidermis, which is the outer-most layer of skin, forms a protective barrier on the surface of the body, consisting of stratified squamous epithelium and preventing the

entrance of pathogens (McGrath *et al.* 2004). The basal layer consists of proliferating cells which despite having a network of keratin intermediate filaments, are relatively undifferentiated (Alonso and Fuchs, 2003). As the basal cells begin to divide, a portion of these cells detach from the basement membrane and start to move outwards, towards the surface of the skin. Therefore, sloughed cells from the skin surface are continuously replaced by the basal cells that are moving towards the surface (Alonso and Fuchs, 2003).

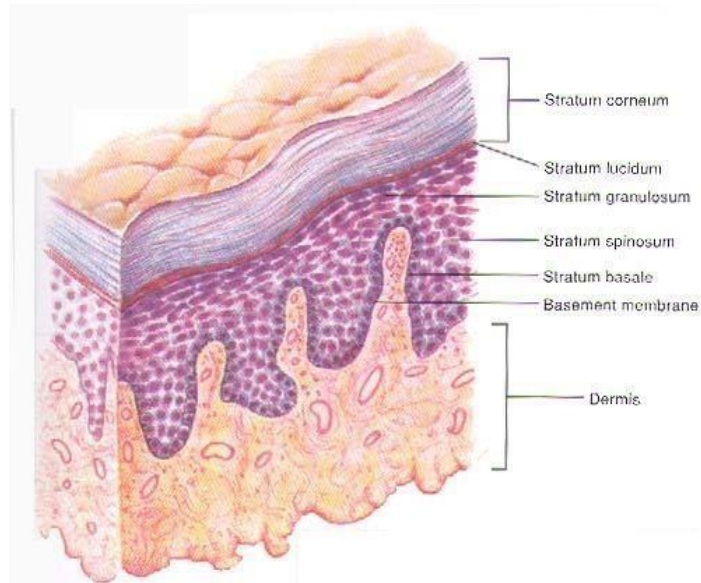


Figure 2.1: The various layers that make up the epidermis (Shier *et al.*, 1999)

2.5.1 Differentiation

Keratinocytes, which constitute 90% of the epidermis, proliferate via mitosis in the basal layer (McGrath *et al.* 2004). Daughter-cells undergo a change in composition during the different phases of differentiation, as they are pushed upwards towards the outermost layer of skin and eventually become anucleated. The phases of this differentiation are represented by the following: 1. The stratum basale, which is the germinal layer of the epidermis, where mitotic activity allows for a constant supply of new keratinocytes. 2. The stratum spinosum, which consists of cells that are undergoing growth and early synthesis of fibrillar proteins. 3. The stratum granulosum, containing intracellular granules that assist in keratinisation. 4. The stratum corneum, comprising flattened cells that have merged and consist of keratin (Pummi *et al.* 2001).

During the differentiation process, keratinocytes form desmosomes between each other as a result of their highly organized configuration. Keratin proteins and lipids are then secreted, which essentially allow for the protective strong exterior of skin (Elias, 1991).

The proliferative and undifferentiated keratinocytes from neonatal as well as adult skin can be used for primary cell culture (Green, 1977). Differentiation of these keratinocytes may be stimulated by the addition of exogenous factors including chemicals such as calcium and Tissue Plasminogen Activator (TPA). Differentiation of keratinocytes can also be stimulated by inhibiting contact with the extracellular matrix, by seeding them onto dermal equivalent substrates (DEDs) that are grown at the air-liquid interface, resulting in epidermal stratification (Lamb and Ambler. 2012).

Although a number of studies have been conducted to develop serum-free, feeder-free media that support the growth and propagation of keratinocytes in 2-dimensional cell culture, the effect that these growth conditions have on keratinocyte stratification and differentiation into 3-dimensional human epidermis remains unknown (Lamb *et al.* 2013). Our study only included Fetal Bovine Serum (FBS).

2.5.2 Terminal differentiation

During the process of proliferation, certain cells in the basal layer are released and begin the cycle of “terminal differentiation” instead. This phenomenon is orchestrated by the interaction between integrins and intracellular signalling pathways (Rippa et al, 2013). In addition, cadherins are receptors that allow for successful adhesion (Stanley *et al.* 1980) and the formation of junctions between cells (Tinkle et al, 2008).

During terminal differentiation, $\beta 1$ integrin activity is downregulated by decreasing the density of integrin receptors on the surface of the cell in combination with the inhibition of transcription of integrin genes and the prevention of immature integrin subunits from undergoing N-linked

glycosylation or being moved to the surface of the cell (Hotchin & Watt. 1992). (Yamamoto *et al*, 2014).

Terminal differentiation remains possible in keratinocytes growing in media supplemented with little to no calcium, leading to involucrin expression, giving rise to the cornified envelope (Watt & Green. 1982). Furthermore, cell surface glycoproteins involved in lectin-binding are also expressed.

In a study by Hoidal & Watt. 1994, it was seen that keratinocytes co-express both integrins and markers of terminal differentiation under conditions where culture medium is supplemented with a low concentration of calcium. When the concentration of calcium is increased, there is a significant reduction in integrins expressed in terminally differentiating cells.

2.5.3 Adhesion

Cell to cell adhesion of keratinocytes is controlled and maintained by various molecules packed into distinct structures the formation of which is regulated by the concentration of calcium ions (Rinnerthaler *et al*, 2015). Under a significantly low calcium concentration (30µm), keratinocytes only develop into a monolayer, lacking adherend junctions or desmosomes. In contrast, when the calcium concentration has been increased, desmosomes are formed, followed by the progression of stratification (Strudwick *et al*, 2015).

Takeichi, 1988 has demonstrated that E-cadherin is involved in the formation of intercellular junctions. Similarly, Mertz *et al*, 2013, demonstrated that cadherin-based cell-cell adhesions has an integral role in orchestrating the physical activity of epithelial cells since naïve mouse keratinocytes without cadherin-based adhesions displayed traction forces that extended throughout the respective colony whereas those with cadherin-based adhesions were able to localize traction forces to the periphery of the colony. This was indicative that cadherin-based adhesions are essential for this mechanical action of keratinocytes.

Desmosomes, which are cell-cell adhesions coupled to the intermediate filament cytoskeleton, are not only essential for the mechanical activity of the cells but are also remodelled to maintain homeostasis of epithelial cells (Osmani and Labousse, 2015).

In a study by Wheelock & Jensen. 1992, interactions shared by E cadherin, P cadherin, Vinculin, $\beta 1$ integrin and desmoplakin were examined, during the development of intercellular junctions and stratification. All markers began concentrating at the cell to cell borders when the calcium concentration was increased.

2.5.3.1 Cadherins

The most commonly expressed cadherins on keratinocytes are P-cadherin, E-cadherin, desmocollin and desmoglein (Caldwell *et al*, 2016).

Van Roy 2014 stated that E-cadherin is expressed in all of the cell layers, but Pcadherin is only expressed in the basal layer, in both in-vivo and in-vitro epidermis.

The binding of cadherins is a homophillic process and is dependent on calcium, seeing that intercellular adhesion is prevented upon the reduction of calcium ions in culture medium during *in vitro* stratification (Strudwick *et al*, 2015). This subsequently prevents keratinocytes from stratifying. Cadherin expression occurs simultaneously with the assembly of desmosomes and adherence junctions when the culture medium has a concentration of $\sim 1.8\text{mM}$ calcium followed by the migration of terminally differentiating keratinocytes to the suprabasal layer from the basal layer (Hennings & Holbrock, 1983).

Interestingly, antibodies to E-Cadherin cause a fractional inhibition of stratification. However, this is not the case with antibodies to integrins (Larjava *et al*. 1990).

As specified in a study by Hodiola & Watt. 1994, a significant loss in integrins is seen in terminally differentiating cells in medium with reduced calcium. This was also seen due to antibodies to P and E cadherin, which prevented stratification, indicating that cadherins downregulate the expression of integrins in keratinocytes. The reason as to

why cadherins allow for the loss of integrins may be because of the competition of the two molecules for a cytoskeletal molecule required for expression in different keratinocytes (Hodivala & Watt, 1994). Since antibodies inhibit integrin mRNA reduction, it can be assumed that this took place at the integrin gene transcription level (Hotchjin & Watt, 1992). This study was in accordance with Wheelock and Jensen, 1992, which reported the inhibition of calcium-assisted stratification by antibodies to E-cadherin and P-cadherin. Furthermore, this study also pointed out that although stratification was substantially reduced in the presence of anti-E Cadherin, there were a few vertical layers that were still formed by the keratinocytes, but they lacked adhesive properties. On the other hand, cells still expressed filaggrin, which is a marker of differentiation. Cells also collected an overload of desmoplanktin at the cell-cell connection, correlating to that of normally stratified *in vitro* keratinocytes.

Interestingly, although stratification and integrin expression were both reduced upon the addition of antibodies to cadherin, desmosomes were still seen between the cells within the subsequent monolayer, indicating that desmosome-formation occurs regardless of the reduction of integrins and vice versa (Hodivala & Watt, 1994).

2.5.4 Stratification

It is possible for naïve keratinocytes to be isolated from the human epidermis and cultured *in vitro* to allow for differentiation and subsequent stratification (Chavez-Munoz *et al*, 2013). This may be done upon the addition of calcium, TPA, limiting the attachment of the keratinocyte extracellular matrix via a forced cell suspension, or by growing keratinocytes onto dermal equivalents at the air-liquid interface, resulting in stratification (Green, 1977) (Lamb & Ambler, 2013).

Prunieras *et al*. 1983 stated that growing primary keratinocytes at the air-liquid interface on dermal equivalents, such as de-epidermalized de-vitalized human skin (DEDs), generated a

structure that closely resembles *in vivo* skin. This may be due to the fact that DEDs retain the extracellular matrix proteins and the original structure of the normal human dermis, resulting in stratified structures of keratinocytes with the same layers present in *in vivo* skin, namely; the basal, spinous, granular and corneum layers. The results of this are concurrent with a study conducted by Lamb & Ambler 2013, who also made use of DEDs to generate a stratified keratinocyte structure.

In vitro methods for propagating primary keratinocytes were developed by James Rheinwald & Howard Green. Briefly, human keratinocytes were seeded onto irradiated J2-3T3 fibroblasts in media consisting of various supplements such as insulin, hydrocortisone, cholera toxin and epidermal growth factor (Rheinwald *et al.* 1975). Recent studies still use the aforementioned techniques, with slight modifications. However, to prevent detrimental consequences such as bovine spongio-encephalopathy and infections with mouse viruses, methods using serum-free, feeder-free conditions were established (Coolen *et al.* 2007).

Bullock *et al.* 2006 described methods developed in the laboratory of Sheila MacNeil, using serum-free media and human fibroblasts, as opposed to murine feeder-cells. As a result, various commercial companies have also headed in the direction of supplying serum-free, feeder-free media that allows for the propagation of keratinocytes in a 2D cell culture environment. Despite successful propagation, the capacity of these keratinocytes to properly stratify under these conditions are limited. The reasons for this are so far unknown.

Lamb & Ambler (2013) used two different serum-free, feeder-free culture media, both of which were successful in providing a suitable *in vitro* environment for primary keratinocyte propagation but these keratinocytes were unable to stratify and develop into a model comparable to *in vivo* skin. Surprisingly, this was not due to a reduced calcium concentration in the media since the increase thereof did not result in an increased capacity of keratinocyte stratification. Upon the addition of 10% Fetal-Bovine Serum (FBS) and calcium to the supplement-free media, keratinocytes began to successfully stratify. Thus, Lamb & Ambler

2013 proved that keratinocytes have the potential to stratify in an *in vitro* provided that they are grown in media supplemented with factors that are biologically active.

Commercially available keratinocytes have various properties that may alter their capacity to stratify and undergo reconstruction. These factors include the passage of the cell type, differences in the methods of isolating the cells, contamination of cell types, differences in the ages of the donor and the body-sites from which the keratinocytes were isolated. Furthermore, keratinocytes which have been passaged more than 3 times have a below-optimum ability to stratify (Gilcrest *et al.* 1983). HaCaTs are capable of being passaged a number of times after the original (Fusenig & Boukamp, 1998).

2.5.5. Keratins

Keratins make up part of the family of intermediate filamentous proteins found specifically in epithelia formed by keratinocytes (Salas *et al.*, 2016). Approximately 30 proteins polymerize into filaments measuring up to 10nm in size, therein lacking auxiliary proteins (; Hatzfeld *et al.*, 1990; Steinert, 1990). Type I keratins are comprised of K9-K20, whereas type II consists of K1-K8. The amino acid sequence is found in an α -helical structure upon the central rod domain of the polypeptides (Coulombe & Fuchs, 1990; Coulombe *et al.*, 1990).

Filament assembly is a process whereby a type-I keratin and a type-II keratin assemble, forming a parallel heterodimer. Thereafter, the rod domains form a coil that subsequently connects with various dimers, resulting in tetramers allowing for the production of protofilaments and eventually, mature filaments (Hermann *et al.*, 2009). In most cases, especially in epithelial tissue specific to a certain differentiation pathway, keratins are seen in particular pairs even though the identity of the type-I keratin forming heterotypic combinations with type-II, *in vitro*, is generally irrelevant in the formation of filaments in fibroblasts. (Moll *et al.* 1982. Banerjee *et al.*, 2014). K5 and K14 are always found in the basal layer, which is mitotically active (Williams *et al.*, 2014) and make their way via the cell cycle to the suprabasal compartment (Fuchs. 1993).

2.5.5.1 The effect of differentiation on keratins

Most often, differentiation in keratinocytes is irrevocable, allowing for a drastic change in the cytoarchitecture and the subsequent flattening of keratinocytes (Wierzbicka *et al*, 2017). Furthermore, the keratinocytes become prone to losing various organelles and their nuclei alike (Anderson *et al*, 2014). The keratins, on the other hand, remain cross-linked to a cornified envelope, consists of keratins inside an insoluble mixture of proteins that are cross-linked by transglutaminases and enclosed by a lipid envelope (Reichert *et al*. 1993. Simon. 1994. Eckert *et al*. 1997. Candi *et al*. 2005). Such changes are followed by the subsequent switch in the expression of keratins, whereby K5/K14 gene expression becomes downregulated in keratinocytes that migrate out of the basal stratum to the surface (Williams *et al*, 2014). The expression of genes coding for a different keratin pair varies in different types of stratified epithelia (Morgan *et al*. 1987).

Characteristically, K1/K10 are expressed in the cornified epithelium but K4/K13 is expressed in non cornified epithelia (Danielsson *et al*, 2014). K3/K12 expression is induced in the cornea (Quinlan *et al*. 1985), whereas K6/K16 expression is detected in the outer root sheath of hair follicles. Interestingly, keratin 15 (Type I) lacks a type-II keratin. (Stark *et al*. 1987. Heid *et al*.1988).

Leube *et al*. 1988 found that the mRNA of K15 is seen in all layers of stratified epithelium, using radioactive in situ hybridization. Essentially, this study, together with various others, implied that K15 is expressed in the basal layer and the expression thereof, will continue, regardless of the upward differentiation of migrating keratinocytes.

Contrastingly, Lloyd *et al*. (1995) demonstrated that K15 is absent in cells in the suprabasal layers and is found only in basal keratinocytes instead.

2.6. HaCaT cells

The HaCaT cell line is an immortalized keratinocyte cell line isolated from adult human skin by Boukamp and colleagues in 1988 (Boukamp *et al.* 1988). The cell line was originally isolated from a healthy skin specimen of male body from the melanoma and the name “HaCaT” is derived from “Human Adult keratinocytes propagated under low Calcium conditions and elevated Temperatures” (Boukamp *et al.* 1988).

The capacity of this cell line to differentiate and stratify has been widely investigated and it was found to express the majority of the markers for differentiation (Fusenig & Boukamp 1988). The cell line lacks tissue organisation when grown in organotypic co-cultures with fibroblasts, however, MaasSzabowski *et al.* (2003) demonstrate that the organization may be restored when the media is supplemented with epidermal growth factor.

2.7. Fibroblasts

Fibroblasts are the prominent cell type seen in the connective tissue of vertebrates, expressing the capacity to synthesize the extracellular connective tissue matrix. Fibrocytes are the metabolically inactive forms of fibroblasts and they play little to no role in the maintenance of the extracellular matrix and tissue (Weissmanhomer *et al.* 1975)

The physical appearance of fibroblasts is dependent on the site from which they were isolated and their respective function and activity. Fibroblasts may retain this function over a number of generations causing them to sometimes stagnate (Weissmanhomer *et al.* 1975).

Fibroblasts are identified by their branched cytoplasm, which encloses a nucleus. Metabolically-active fibroblasts have a substantially large amount of rough endoplasmic reticulum. Active fibroblasts are spindle-shaped with much fewer endoplasmic reticula. Over a wide surface area. Fibroblasts are incoherent, but become locally aligned in parallel clusters when confluence is reached (Dave & Bayless, 2014).

Fibroblasts have the capacity to synthesize a number of components vital for the structure and maintenance of the extracellular matrix, namely; collagens, glycosaminoglycans, reticular and elastic fibers and glycoproteins (Weissmanhomer *et al.* 1975).

2.8. Stromal interactions

An interaction between the neighbouring cells and the extracellular components of the neighbouring stroma are vital for the replenishment and restoration of epithelial tissue. A demonstration of such is seen in studies regarding wound-repair and transplants which support observations that tissue repair and replenishment is dependent on the action of cytokines and growth factors (Luger & Schwarz. 1990).

Similarly, Rheinwald & Green 1975, showed that the proliferation and differentiation of keratinocytes, *in vitro*, can be induced and supported by stromal cell interactions. This concept is used in 2D feeder layer organotypic co-cultures with post-mitotic fibroblasts.

It is important to note that the proliferation of keratinocytes in such cases is not only supported by the influx of growth factors provided by the fibroblasts, but rather by the continuous interaction between both cell lines in a double paracrine mechanism (Maas-Szabowski *et al.* 2001). This allows for keratinocytes to maintain the production of growth factors in fibroblasts by the secretion of interleukin-1 (IL-1) α and β , which in turn, induce the expression of keratinocyte growth factor/fibroblast growth factor 7 (KGF/Fgf-7) and granulocyte macrophage colony-stimulating factor (GM-CSF). These growth factors allow for the proliferation of keratinocytes and their expression thereof is increased during wound repair (Maas-Szabowski *et al.* 2000)

Maas-Szabowski *et al.* 2000 proved that the double paracrine mechanism between the two cell types allows for the release of IL-1 by keratinocytes and KGF/FGF-7 and GM-CSF produced by fibroblasts, thus inducing differentiation and stratification of keratinocytes when using post-mitotic fibroblasts as a feeder layer in organo-typic co-cultures of the two cell types. The study demonstrated that these molecules constitute part of the epithelial-stromal interactions that induce and maintain tissue-repair. The study also corroborated that both growth factors are not functional in the absence of stromal cells.

Maas-Szabowski & Fusenig (1996) showed that it is especially advantageous to use post-mitotic fibroblasts as opposed to naïve fibroblasts as it will eliminate the proliferative action of fibroblasts in the co-culture, thus omitting inconsistent stromal cell numbers. Fibroblasts may become post-mitotic when exposed to γ -irradiation.

In contrast, Blanton *et al* 1991 stated that human keratinocytes involved in organotypic co-cultures did not stratify and differentiate which is in keeping with studies by Durst *et al.* 1989, Oda *et al.* 1996 and Tsuenenganga *et al.* 1994. However, a substantial number of later studies looking into stratification, indicate that immortalized human keratinocytes have a high capacity for stratification (Boukamp *et al.* 1998).

Organotypic co-cultures comprising of HaCaT cells and fibroblasts display minimum differentiation and stratification and suffer abnormal structure and keratinization (Haake & Polakowska. 1993). Comparably, HaCaT cells have the capacity to form stratified, differentiated structures, taking a much longer time to do so as opposed to normal keratinocytes. This delay is primarily due to the decreased secretion of IL-1 in HaCaT cells, resulting in a reduced expression of growth factors, KGF and GMCSF. Furthermore, the receptors of both growth factors experience a substantial decrease in expression resulting in the loss of signal transduction. In addition, in HaCaT cells there is a reduced release of the keratin growth factor, TGF- α . Thus, by adding TGF- α , HaCaT cells may regain the ability to stratify and differentiate. In addition, there is also a simultaneous increase in IL-1 and the subsequent increase in KGF and GM-CSF, subsequently resulting in a functional interaction with stromal cells in effort to generate a stratified skin model from HaCaT cells comparable to normal keratinocytes (Maas-Szabowski *et al.* 2003).

CHAPTER 3 – MATERIALS & METHODS

3.1. Cell culture

3.1.1. Cell lines

For the propagation of *C. trachomatis*, the McCoy cell line (ATCC® number CRL – 1696) was used. This is an immortalised, adherent mouse fibroblast cell line, commonly used for the propagation of chlamydia.

A spontaneously immortalized human keratinocyte cell line, known as the HaCaT cell line, was used for the generation of the stratified keratinocyte model. The cell line was donated by Professor Norbet E. Fusenig of the Cancer Research Centre, Heidelberg, Germany).

3.1.2. Cell Culture Media

3.1.2.1. McCoy cells

For the McCoy cell line, Eagle's Minimum Essential Medium (EMEM) (BioWhittaker™, Walkersville, USA) supplemented with Earle's Balanced Salt Solution (EBSS), HEPES (25mM) (BioWhittaker™, Walkersville, USA) and L-glutamine (2mM) (BioWhittaker™, Walkersville, USA) was used. HEPES buffer assists in maintaining the pH at 7.4. Glutamine, is separately added to the media since it breaks down faster in complete media compared to other amino acids (ATCC technical information).

3.1.2.2. HaCaT cells

HaCaT cells are a spontaneously immortalized human adult skin keratinocyte cell line developed by Boukamp and team in 1988 (Boukamp *et al.* 1988). This cell line has been nontumorigenic for over 300 passages, which spans over 6 years in culture (Boukamp *et al.* 1994). The cell line is capable of expressing all epidermal differentiation markers, even though it undergoes various chromosomal changes whilst adapting to autonomous growth *in vitro* (Boukamp *et al.* 1988). When grown in organotypic cocultures with fibroblasts, HaCaT cells display little to no tissue organization, however, this is restored when supplemented with epidermal growth factor (Boukamp *et al.* 1998).

The cell line is widely studied in cell-cycle research (Chaturvedi *et al.* 1999), carcinogenesis (Boukamp *et al.* 1994) and they are susceptible to apoptosis (Lee *et al.* 2005).

For this study, HaCaT cells were propagated in Hyclone Dulbecco's Modified Eagle's Medium (DMEM) supplemented with HEPES (10mM) and used as a result of lab-availability. Sterile heatinactivated fetal bovine serum (FBS) (Gibco™, Scientific group) was added to the media to provide various cytokines and growth factors required for the regulation of cell propagation, growth and differentiation.

For the development of the stratified keratinocyte model, HaCaT cells were grown in Hyclone DMEM supplemented with both, 10% FBS and 1.4 mM CaCl₂.

Upon confluency, cells were exposed to the air-liquid interface, where FBS and CaCl₂ supplemented KGM-Gold™ Keratinocyte Growth Medium with Bulletkit™ (Lonza) was added to the bottom chamber. Bullet kit™ contains hydrocortisone (0.5ml), bovine pituitary extract (BPE) (2ml), human recombination epidermal growth factor (rhEGF) (0.5ml), transferrin (0.5ml), epinephrine (0.25ml), GA-1000 (0.5ml) and insulin (0.5ml).

Incubation of cultures for all experiments were carried out in an incubator with 5% CO₂ in 95% air, either at 33°C or 37°C.

3.1.3. Cultivation and revival

Cells suspended in storage media in cryovials were kept at -70°C for short term storage or at -96°C for long term storage. When required, cells were thawed by placing the cryovial in a beaker with water at 37°C. Thereafter, the cryovial was gently swirled, the outside swabbed with 70% ethanol and moved into a class II biosafety cabinet where cells were seeded in a 75 cm² tissue culture flask with the appropriate cell culture medium with a temperature of 37°C. Thereafter, the flask was incubated at 37°C with 5% CO₂.

To observe confluency of the cells and to determine possible contamination or cell deterioration, cells were viewed with an inverted microscope every day.

Media was changed every other day, where spent media was discarded and cells were washed 3 times with phosphate buffered saline (PBS) (Dulbecco A Oxoid) with Ca²⁺ or Mg²⁺ pH 7.2 to eliminate residual FBS and cells that were not attached. Thereafter, fresh cell culture media, supplemented with 10% FBS, was added to the flask, followed by reincubation.

Subculturing was conducted when the monolayer reached 90% confluency. Cells were seeded into new culture flasks to maintain stock, in 24-well plates for experiments or in cryovials for storage. The subculturing technique remains the same for all cell lines, except for HaCaT cells, which need one extra step. After washing the keratinocyte monolayer 3 times with PBS, pH 7.4, 2ml 0.05 ethylenediaminetetra-acetic acid (EDTA) solution (Sigma, Steinheim, Germany) is added to the flask and incubated for 5-10 minutes at 37°C. Once intracellular spaces were microscopically detected, EDTA was removed.

After this step, the procedure remains standardized for all cell lines. One ml 0.05% trypsin – 0.02% Versene (EDTA) solution (BioWhittaker™) in PBS, pH 7.4. is added. The flask is then gently swirled, ensuring total coverage of the surface area of the flask. After incubation for 1 minute at 37°C, the flask is gently tapped to assist detaching of the cells. Thereafter, 1ml undiluted FBS is added to the flask to neutralize the trypsin. This was done with a Pasteur pipette, by squirting FBS against the back of the flask, thus removing detached cells. The cell suspension was decanted to be seeded or cryopreserved.

For cryopreservation, cell suspensions with an equal volume of storage fluid (Appendix), which was added drop by drop to the suspension, was aliquotted into the cryovials and sealed with parafilm. The name of the cell–line, storage date, passage number and the cell concentration was recorded on each cryovial.

3.1.4. Determining the cell numbers

In order to deduce the number of viable and non-viable cells, the Trypan Blue Dye Exclusion Assay was conducted, using a Neubauer haemocytometer.

Once it was wiped with 70% ethanol, the haemocytometer was covered with a square coverslip and 20 µl cell suspension was added to 20 µl 0.4% trypan blue solution (Sigma) and thoroughly mixed. A drop of the mixture was then placed at the edge of the coverslip, where it was drawn in to fill the counting chamber. Viable and dead cells are easily differentiated when viewing under a light microscope, since trypan blue remains in dead cells and is excluded from viable cells, allowing dead cells to appear blue and viable cells colourless. The number of clear and blue cells were counted in the 5 primary squares (Maleka *et al.* 1996). Clumped cells were counted as though they were separate individual cells. If too many clumps were seen, the suspension was mixed again and the procedure was repeated. Since the volume of each primary square is 1×10^{-4} ml, the following formula was used to calculate the concentration of cells per ml:

$$\text{Concentration} = N/5 \times 10^4 \times \text{dilution factor}$$

Where N = total number of cells in the 5 primary squares.

The total cell number was calculated by the following:

$$\text{Total cell number} = \text{concentration (cells/ml)} \times \text{total volume (ml)}$$

The percentage cell viability was calculated as follows:

$$\% \text{ cell viability} = (\text{Number of viable cells}) / (\text{Total number of cells}) \times 100$$

On the occasion that the number of cells in each primary square was too many to count, the cell suspension was diluted with PBS and the Trypan Blue Exclusion Assay was repeated.

3.2. Bacterial culture

Chlamydia trachomatis L2 strain 434 (ATCC® number VR-902B™) was used for this study. This strain was originally isolated from a bubonic aspirate of a military service man returning from Asia who presented with typical LGV symptoms (Schachter *et al.* 1969).

3.2.1. Propagation and storage of *C.trachomatis*

McCoy cells were cultured in 75cm² tissue culture flasks and passaged according to the procedure described above. The cell cultures were incubated at 37°C with 5% CO₂ until the monolayer of cells reached 80-90% confluency. Confluency and possible potential contamination was assessed by viewing the cells on a daily basis with an inverted microscope. Media was changed every second day.

When the desired confluency was reached, spent media was aspirated from the flask. Thereafter, the monolayer was washed three times with PBS. Twenty µl Chlamydia Growth Medium for propagation (CGM-P) was added to the flask. CGM-P consists of EMEM (BioWhittaker™) with Earle's Balanced Salt Solution (EBSS) (Gibco®), non-essential amino acids (NEAA) and sodium pyruvate, supplemented with 10mM HEPES, 2mM L-glutamine, 1µg/ml cycloheximide, 5.4 mg/ml glucose, and 10% FBS. In addition, gentamicin (10µg/ml), and amphotericin B (5µg/ml) were added.

Chlamydial elementary bodies suspended in 100 µl sucrose-phosphate buffer (SPG) were added to the flask. A flask to which 100 µl SPG without chlamydia was added served as the negative control. Flasks were centrifuged for 1 hour at 1200g at room temperature in a temperature controlled centrifuge and incubated for a further hour at 37°C. A media change with CGM-P supplemented with 10% FBS was then conducted to remove any dead and unattached cells. The flasks were incubated at 37°C for 48 hours.

3.2.2. Propagation of chlamydia

After 48 hours, the cultures were viewed with the inverted microscope to detect chlamydial inclusions. CGM-P media was removed from the flasks and the monolayers were washed with PBS. SPG supplemented with 10% FBS was added as well as sterile glass beads. The flasks were vortexed to disrupt the McCoy cell monolayer, thus releasing chlamydial EBs. The cell lysate containing chlamydial EBs was transferred to a clean centrifuge tube. Cell debris was pelleted by centrifugation at 1000 x g

for 10 minutes at 4°C. The supernatant containing the chlamydial EBs was transferred to a clean centrifuge tube, aliquoted and frozen at -80°C. The pellet containing cell debris was discarded.

3.2.3. Determination of the infectious titre

Serial 10-fold dilutions of the *C. trachomatis* isolate were prepared and used to inoculate McCoy cells grown in a 96-well microtitre plate to 80% confluency. Cells were centrifuged at 1200 x g for 1 hour at room temperature and incubated at 37°C for 1 hour. Spent media was removed and replaced with fresh CGM, followed by further incubation at 37°C in 5% CO₂. After incubating for 48 hours, infection of the monolayers was confirmed using the MicroTrak® *C. trachomatis* Culture Confirmation Test Kit.

This kit involves contains monoclonal antibodies against an antigenic determinant of the major outer membrane protein (MOMP) found in the outer membrane of all known serovars of *C. trachomatis*. These antibodies are labelled with FITC. *C. trachomatis*-positive cultures will be identified by the presence of green fluorescing intracellular chlamydial inclusions, against a background of red cells due to the Evans Blue counterstain. Assuming that each inclusion body evolves from one EB, the number of inclusions can be enumerated per field of view, allowing for the determination of the infectious titre. The required calculation for determining the infectious titre is as follows:

Conc. (CFU/ml) = (# of inclusions/n) x (100ul/V) x (C x D) Where:

- n = # of fields counted
- V = Volume of the inoculum (ml)
- C = Objective lens conversion factor
- D = Dilution factor (ml)

3.3. Preparation of a stratified keratinocyte model

A vial of stored HaCaT cells was revived as per 3.1.3. Once the cells were 80% confluent, they were trypsinised and seeded into 24 well plates, in which polytetrafluoroethylene (PTFE) membrane inserts (Pore size 3µm) (Costar) had been placed. Cells were seeded at a cell density of 1×10^6 cells/ml.

A volume of 600µl cell culture medium (Hyclone DMEM) supplemented with 10% FBS was added at the bottom chamber, whereas 100 µl cell suspension in culture medium (Hyclone DMEM) supplemented with 10% FBS added to the upper chamber. Cells were then incubated at 37°C with 5% CO₂ and a media change was conducted every second day.

Upon confluency, cells were exposed to the air-liquid interface by replacing medium in the bottom chamber with KGM-Gold media prepared according to the manufacturer's instructions. This medium was supplemented with 10% FBS and the CaCl₂ concentration was raised from 0.1mM to 1.4 mM to facilitate stratification and differentiation (Lamb and Ambler, 2013).

Thereafter, inserts were incubated at either 33°C or 37°C for 2 weeks and 3.5 weeks.

Controls were also prepared as outlined above. These cells were not exposed to the air-liquid interface and were incubated for 1 week and at both temperatures.

3.4. Infection of the stratified keratinocyte model with *C. trachomatis*

Spent media was discarded and fresh CGM-P was added to all wells. The volume of fresh media added was 600 µl to the bottom chamber, and 50 µl to the upper chamber. *C. trachomatis* L2 elementary bodies (infectious titre: 1.5×10^9 cells/ml) were prepared in SPG with FBS to a concentration of 4×10^7 EB/ml.

Fifty µl of the EB suspension in SPG-FBS was added to the upper chamber and the plates were incubated at 33°C and 37°C respectively for 3 hours. Thereafter, media was removed from the upper chambers followed by incubation at their respective temperatures for a further week to allow for penetration to the underlying layers.

Cycloheximide was omitted from the medium, since it would negatively affect eukaryotic cell replication, thus preventing an *in vivo*-like environment (Elela *et al.* 1997).

Control wells seeded with 50 µl SPG with FBS in the upper chamber instead of chlamydia suspension were incubated at both 33°C and 37°C.

3.5. Histology

Various histology techniques were used to compare whether or not a stratified layer of keratinocytes was formed and how closely the *in vitro* system mimicked human skin *in vivo*. Histological observations were used to select the temperature and duration of incubation that allowed keratinocytes to differentiate and most closely approximate the structure of the *in vivo* epidermis for use in future experiments.

Once the system was optimised, the cells were infected with *C. trachomatis* and stained with the MicroTrak® *C. trachomatis* Culture Confirmation Test Kit to confirm infection and track the migration of *C. trachomatis* through the layers. Hematoxylin and Eosin (H&E) staining and immunohistochemistry was performed on infected cell cultures to detect structural changes.

For histology, cells were fixed, embedded in a paraffin block, cut in thin sections, stained and visualised using an appropriate microscope.

3.5.1. Preparation of Costar® Transwell® Inserts for histology

In order to prepare the Transwell inserts for histology, a protocol by Corning Incorporated (Corning New York) was used. The procedure was carried out with minor modifications as follows: Transwell membranes were rinsed with Hank's Buffered Saline Solution (HBSS) (37°C). The HBSS was added slowly and aspirated carefully thereafter so that neither surface of the membrane was touched. The membrane is extremely delicate therefore it was of utmost importance that it was handled with care, to prevent shredding or tearing. The rinse procedure was repeated.

Thereafter, 10 % phosphate buffered formalin (pH 7.4) brought at room temperature was added to the wells and membrane inserts. The plate was then left in the biosafety cabinet at room temperature to

allow for fixation. After 1 hour, the membrane inserts were rinsed with HBSS (37°C). The inserts were then dehydrated by replacing the HBSS with ethanol in a graded series of concentration, as follows:

35% ethanol for 10 minutes; 50% ethanol for 10 minutes; 70% ethanol for 10 minutes; 95% ethanol for 10 minutes; 100% ethanol for 10 minutes and 100% ethanol for 10 minutes.

The 100% ethanol was then replaced with Isopropanol. The isopropanol was left to infiltrate the cells and membranes for 10 minutes and this step was repeated. Once the isopropanol was removed from the wells and membrane inserts, liquid paraffin (58°C) was added. The plate was then placed in an oven (58°C) for 1 hour. Thereafter, the paraffin was changed and left in the oven for a further hour. The plate was then removed from the oven and the paraffin was allowed to solidify. By warming the outside of the plate, the inserts were removed whilst still containing solid paraffin on the inside of the inserts.

For each step of this procedure, the volume of the reagent added to the wells remained standardized: 600 µl at the bottom chamber and 100 µl at the upper chamber.

Using a small scalpel blade that had been wiped with 70% ethanol, the membrane (with attached paraffin) was cut from the insert. The paraffin embedded membrane was then placed into a paraffin boat. The plug was held in a vertical, upright position using a clean pair of forceps whilst the paraffin was poured over it. This orientation allowed for a cross section view of the cells after sectioning.

Thin sections of 8 µm of the paraffin-embedded membrane were cut according to standard histology procedure using a microtome. The sections were mounted on glass slides and left to dry.

3.5.2. Hematoxylin and Eosin staining

H&E staining was used to determine the resemblance of the stratified keratinocytes to *in vivo* human skin. This staining technique was also used after infection with *C. trachomatis* (see below), in addition to the MicroTrak® *C. trachomatis* Culture Confirmation Test Kit. The haematoxylin stains the nuclei of cells, causing them to appear a distinct shade of blue. The counterstaining with a solution of eosin Y (alcoholic or aqueous) causes other eosophilic structures to appear red, pink or orange.

H & E staining was performed as follows using the method described by Luna (1960):

Thin sections were deparaffinised. This was done using a series of decreasing concentration of ethanol. Slides were dipped in 100% ethanol followed by 90% ethanol, 70% ethanol, 50% ethanol, 30% ethanol and distilled water. Mercuric chloride crystals were removed by dipping slides in iodine. Slides were then flooded with Mayers hematoxylin for 15 minutes. Thereafter, they were washed with running tap water for 20 minutes and counterstained with eosin for 1 minute. Lastly, the thin sections were dehydrated in 95% and 100% alcohol, in order to remove the excess eosin. The sections were then visualised using a bright field microscope.

3.5.3. Immunofluorescence

Immunofluorescence was used to determine whether or not the keratinocytes had differentiated. Mouse derived primary antibodies against keratin 10, keratin 14 and involucrin were used. A goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC) was used to facilitate detection. The antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, U.S.A).

After staining, thin sections were mounted using Prolong Gold anti-fade with DAPI and viewed and photographed using the Evos™ FL Cell Imaging System with appropriate filters.

The protocol for immunofluorescence staining was carried out according to the manufacturer's instructions with minor optimizing modifications. The procedure was conducted as follows:

Thin sections were deparaffinised in xylene. Although the protocol suggests three changes for five minutes each, the slides were dipped in xylene once for approximately 30 seconds only. This was done because the 3 x 5 minutes were too harsh on the sections as they were being washed away during the trial run.

The sections were then hydrated gradually through graded alcohol. The original protocol suggested washing in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each and finally washing in deionized water for 1 minute with stirring. However, since this was also found to be

too harsh during the trial run, the slides were only dipped in the respective concentrations of alcohol for approximately 30 seconds each.

Antigen unmasking was then conducted by placing the slides in a container with enough 10mM Sodium Citrate Buffer, pH 3.5 with 0.01% (w/v) EDTA to cover the slides. The container was then placed in a water bath at 95°C for 5 minutes. It was then topped off with fresh buffer and left in the water bath for an additional 5 minutes. Slides were then allowed to cool in the buffer for 20 minutes and washed in deionized water three times for two minutes each. Excess liquid was then aspirated from the slides.

Slides were then incubated at room temperature with blocking serum (10% goat serum in PBS) for 20 minutes. This allows for the suppression of non-specific binding of secondary antibodies. Blocking serum is derived from the same species in which the secondary antibody is raised. After 20 minutes, the slides were washed with PBS.

A 1:50 dilution was chosen for each primary antibody (200 µg/ml) in PBS with 0.15% blocking serum. The slides were then incubated with the diluted primary antibody (4µl/ml) for 60 minutes at room temperature. Thereafter, slides were washed three times with PBS.

The slides were then incubated for 45 minutes in a dark chamber with FITC-conjugated secondary antibody diluted in PBS with 1.5% blocking serum. Thereafter, slides were washed three times with PBS. This step needed optimisation which is discussed in chapter 4.

To confirm that non-specific binding of the secondary antibodies had not occurred, slides with cells incubated at either 33°C or 37°C were prepared as per the procedure outlined above whilst omitting the step involving incubation with the primary antibody.

Coverslips were then mounted onto the slides using Prolong Gold Antifade with DAPI and viewed with the Evos™ FL Cell Imaging System and appropriate filters.

CHAPTER 4 – RESULTS

4.1 Optimisation of methodology

The methodology applied in our experiments is based on publications on similar work with keratinocytes. Some aspects had to be optimized for working with HaCaT cells. This included the duration of incubation, the working dilutions of primary and secondary antibodies used for immunofluorescence tests and keratinocyte-fibroblast organotypic co-cultures.

4.1.1 Duration of incubation

In order to determine which duration of incubation at both 33°C and 37°C, was sufficient for developing stratified keratinocytes, HaCaT cells were grown in DMEM with 10% FBS on collagen coated PTFE Transwell membrane inserts. Upon confluency, which was reached after approximately 11 days of incubation at 37°C, cells were exposed to the air-liquid interface by removing the media from the upper chambers and replacing the spent media in the bottom chambers with KGM-Gold media supplemented with 10% FBS and 1.4mM CaCl₂. The wells containing the inserts were then incubated at 33°C and 37°C for 14 days and 24 days Fresh media was fed from the bottom chamber every other day.

Inserts were then embedded in paraffin, cut in thin sections (3.5.1) and stained with H & E stain (3.5.2).

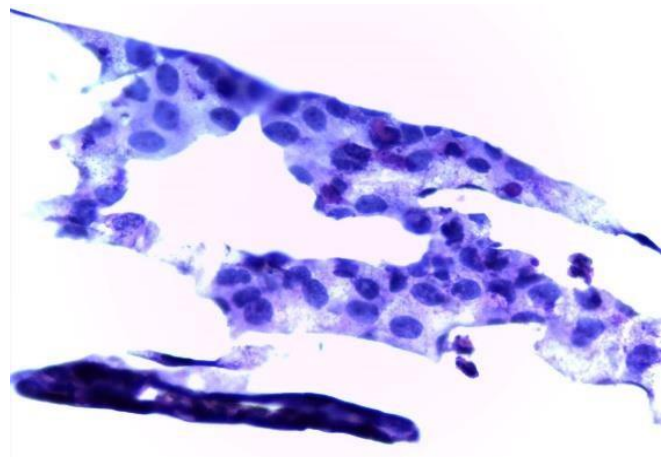
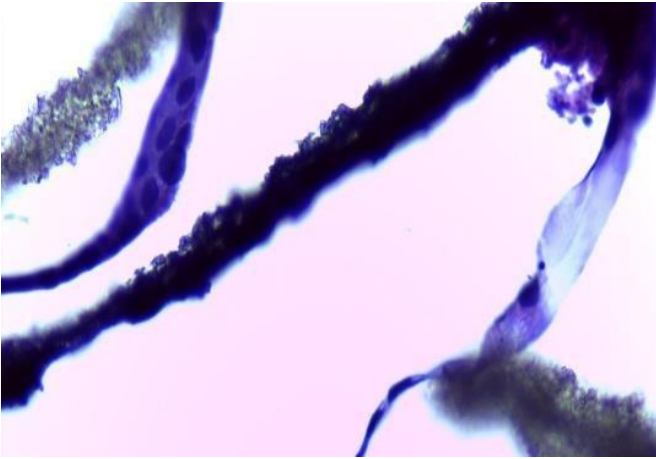
Figure 4.1 shows the extent of stratification that had occurred in keratinocytes after 14 days of incubation at 33°C and 37°C compared to the keratinocyte stratification observed after 24 days of incubation at the same temperatures. Figure 1 shows that keratinocytes have the potential to stratify at 33°C, regardless of the duration of incubation since more than one layer is depicted.

Although stratification is seen after 14 days of incubation at 33°C and 37°C, the layering is more distinct and visible after incubation for 24 days at both temperatures. In addition, the keratinocyte layers that had formed after 24 days were more intact, looked more complete and resembled *in vivo* skin closer than those that were produced after only 14 days.

14 days

24 days

33°C



37°C

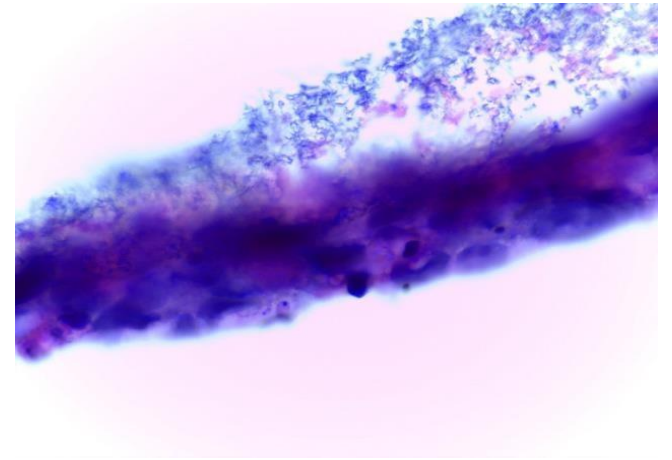
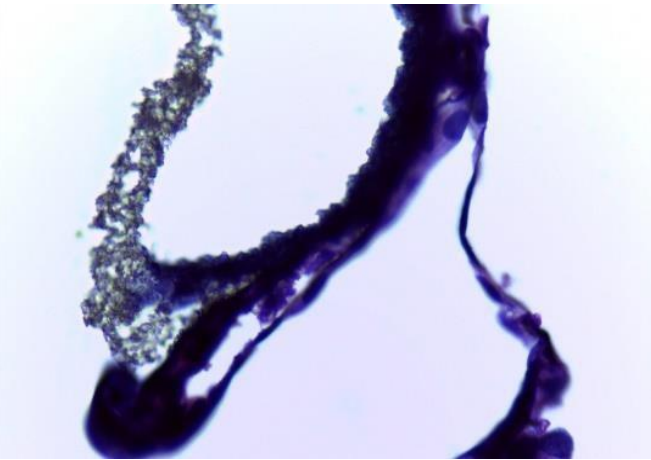


Figure 4.1: Comparison of differentiation of HaCaT cells grown at 33°C and 37°C for 14 days and 24 days (X1000).

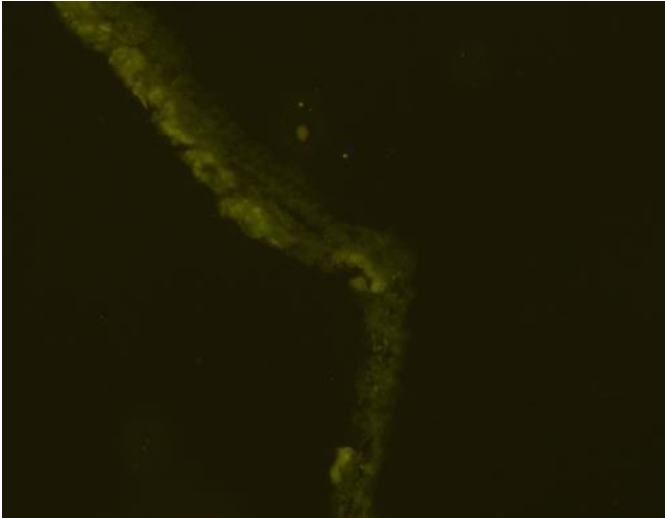
4.1.2 Dilutions of primary and secondary antibodies for Immunohistochemistry

Cells grown at the air-liquid interface at 37°C for 24 days were prepared, cut into thin sections and placed on Apex BOND adhesive slides (Leica Biosystems Inc. Illinois, USA). Thereafter, the sections were prepared for immunohistochemistry (3.5.3) and stained with antibodies to Keratin 10, Keratin 14 and Involucrin. Dilutions of 1:50 and 1:500 were prepared for the primary antibodies, whilst 1:100 and 1:400 dilutions of FITC-conjugated secondary antibody were prepared. The optimal combination of dilutions of both antibodies was then determined.

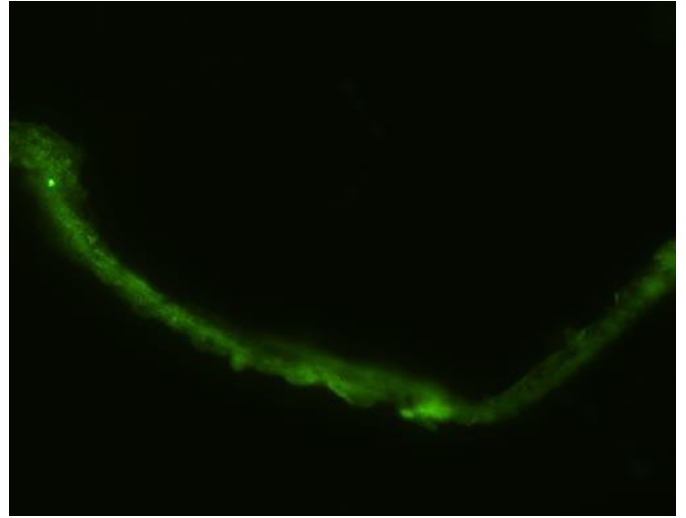
Figure 4.2A shows that fluorescence was detected after staining with the different combinations of antibody dilutions. For keratin 10 detection a 1:50 dilution of the primary antibodies and a 1:400 dilution of the secondary antibodies gave the brightest fluorescence and was therefore used in experiments.

The different combinations of antibodies to detect keratin 14 and involucrin all produced similar levels of fluorescence. However, the involucrin antibody combinations showed brighter fluorescence except for the combination of the highest dilutions. This is shown in Figures 4.2A and 4.2B. In the experiments the lowest dilutions of both antibodies were used for the detection of both.

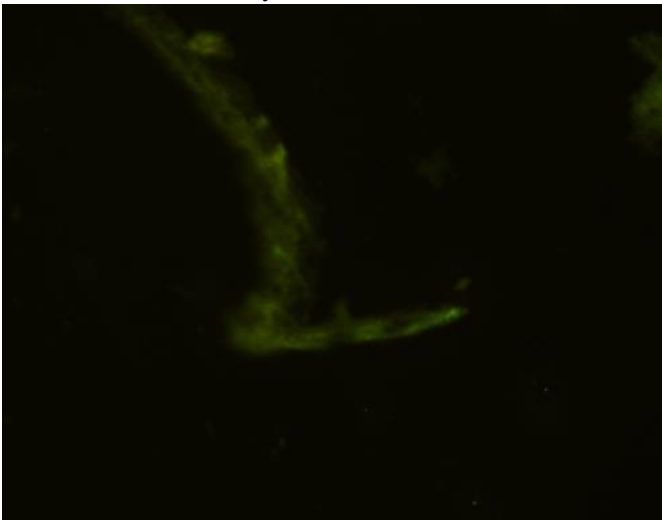
Anti-Keratin 10 1:50 FITC-
conjugated secondary antibody
1:100



Anti-Keratin 10 1:50 FITC-
conjugated secondary
antibody 1:400



Anti-Keratin 10 1:500
FITC-conjugated secondary
antibody 1:100



Anti-Keratin 10 1:500
FITC-conjugated
secondary antibody 1:400

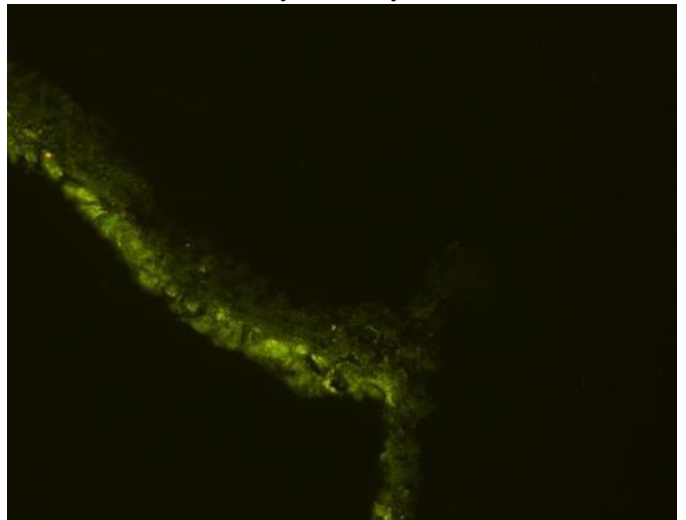
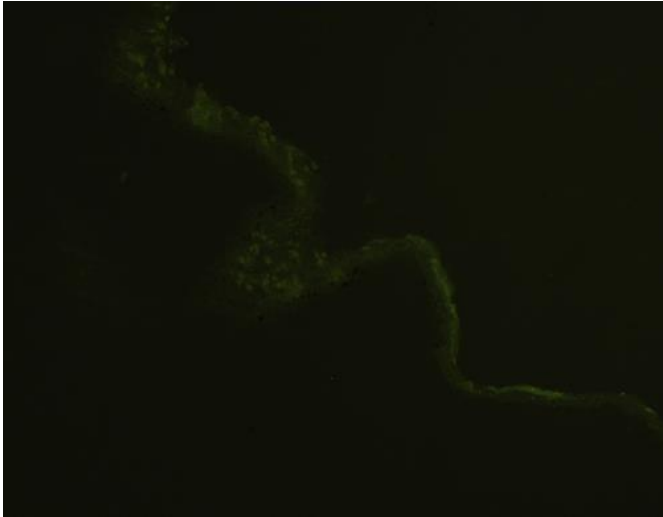


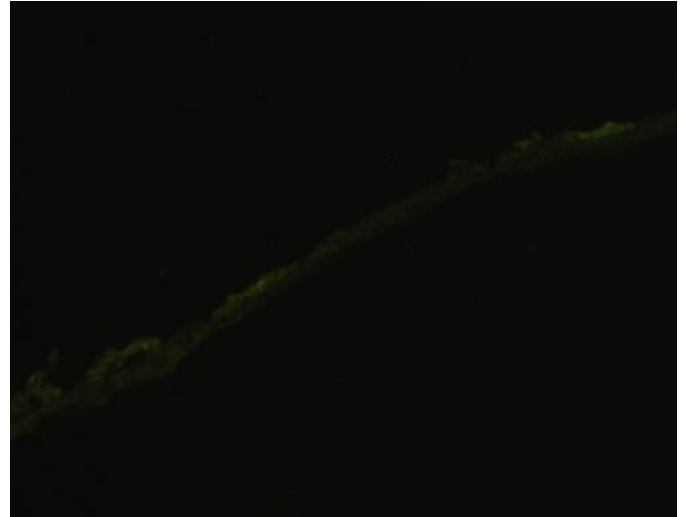
Figure 4.2A: Comparison of fluorescence produced after staining thin sections of layered keratinocytes with various combinations of dilutions of primary and secondary antibodies to keratin 10 (X40).

Anti-Keratin 14 1:50 FITC-
conjugated secondary antibody
1:100

Anti-Keratin 14 1:50
FITC-conjugated secondary antibody
1:400



Anti-Keratin 14 1:500
FITC-conjugated secondary
antibody 1:100



Anti-Keratin 14 1:500
FITC-conjugated secondary
antibody 1:400

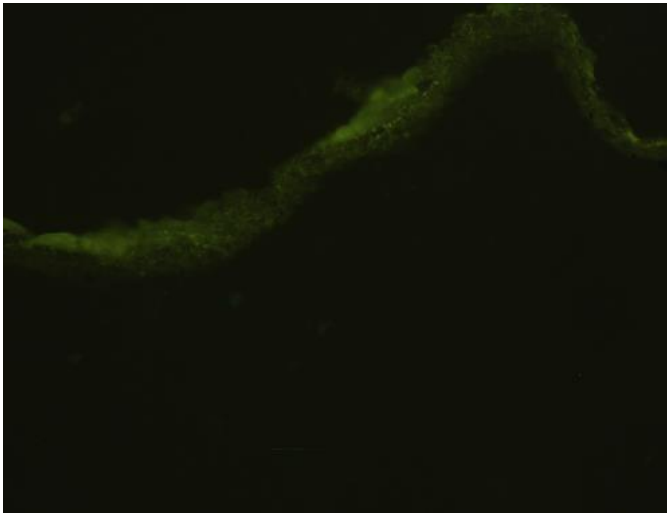


Anti-Involucrin 1:50 FITC-
conjugated secondary antibody
1:100



Anti-Involucrin 1:50 FITC-
conjugated secondary
antibody 1:400

Figure 4.2B: Comparison of fluorescence produced after staining thin sections of layered keratinocytes with various combinations of dilutions of primary and secondary antibodies to keratin 14 (X40).



Anti-Involucrin 1:500
FITC-conjugated secondary
antibody 1:100



Anti-Involucrin 1:500
FITC-conjugated secondary
antibody 1:400

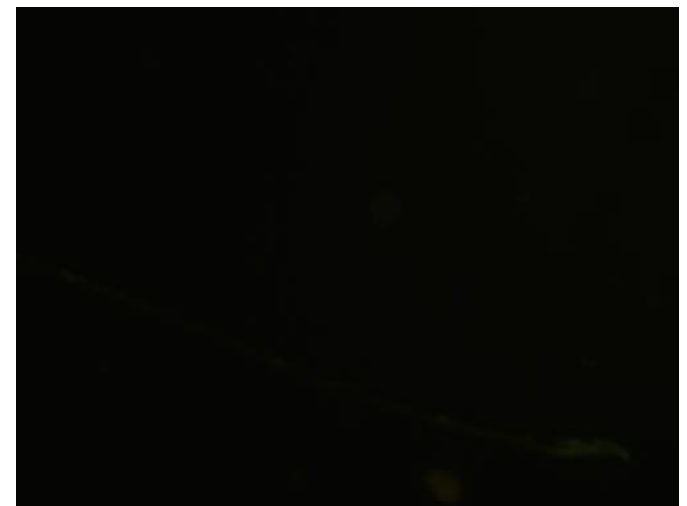


Figure 4.2C: Comparison of fluorescence produced after staining thin sections of layered keratinocytes with various combinations of dilutions of primary and secondary antibodies to involucrin (X40).

4.1.3 The use of organotypic co-cultures grown on a collagen matrix as an additional and alternate method to generate a stratified keratinocyte structure

Co-cultures of HaCaT cells and fibroblasts to generate a stratified *in vitro* model were performed as previously described by Stark *et al.*, 1999. Briefly, collagen type 1 (4mg/ml) from rat's tail (Sigma) was prepared by dissolving it in 0.1% acetic acid and mixing it with 10X HBSS. The pH of the mixture was then neutralized with 2M NaOH to pH 7. MRHF Human Dermal Fibroblasts were treated with mitomycin (0.4mg/ml) for 4 minutes at 37°C in order to halt replication. The non-dividing fibroblasts were trypsinized, enumerated in a haemocytometer, and mixed with the prepared collagen solution to reach a concentration of 6×10^5 cells/ml. This fibroblast suspension in collagen solution was cast onto polycarbonate Transwell filter inserts with pores of 3 μ m diameter (Costar®). After 24 hours, HaCaT cells were seeded (1×10^6 cells/ml) onto the fibroblast containing collagen type 1 gels in the Transwell inserts. These co-cultures were incubated for 24 hours at 33°C and 37°C, after which media was replaced with DMEM supplemented with 10% FBS and 50 μ g/ml L-ascorbic acid. Media was replaced three times per week.

Once confluent, cells were exposed to the air-liquid interface and fed with DMEM supplemented with 10% FBS, 50 μ g/ml L-ascorbic acid, 2 μ g/ml TGF- α (R&D Systems) and 5 μ g/ml L-1 (R&D Systems). Once exposed to the air-liquid interface, inserts would have been incubated at their respective temperatures for an intended period of 24 days.

It is for this reason that we reverted to commercially available collagen coated inserts, as seen in this study.

Figure 4.3 shows that after just two weeks of incubation at 33°C and 37°C, the collagen present in the inserts had shrunk. HaCaT cells seeded onto the collagen and fibroblast mixture had not even reached confluency before the shrinkage had occurred. The collagen is seen as the white circles situated inside the inserts of the wells. The collagen is supposed to cover the surface area of the membrane insert for the entire incubation period. However, it was found that the collagen only covered a fraction of the

membrane. The level of shrinkage of the collagen on the inserts incubated at 37°C was more than on those incubated at 33°C.

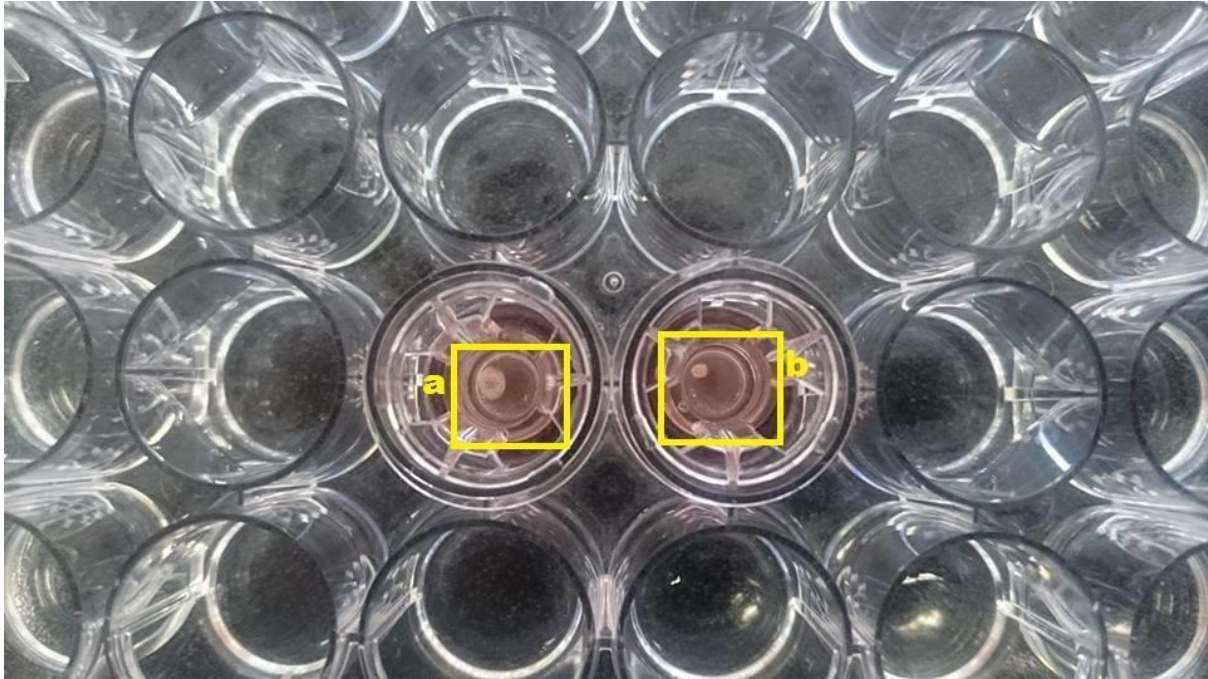


Figure 4.3: Collagen shrinkage in the fibroblast-keratinocyte co-cultures after 14 days of incubation at 33°C (a) and 37°C (b).

4.2 Infection of stratified keratinocytes grown at 33°C and 37°C with *Chlamydia trachomatis*

HaCaT cell cultures on commercially available collagen coated PTFE Transwell membrane inserts were prepared as described in 3.3. Upon confluency, after approximately 11 days, cells were raised to the air-liquid interface and spent media in the bottom chamber was replaced every other day with fresh KGM-Gold media supplemented with 10 % FBS and 1.4 mM CaCl₂. The cells were then incubated for 24 days at 33°C and 37°C. The HaCaT layers were then infected with *C. trachomatis*, L2 strain and incubated for a further week (3.4.).

The membrane inserts were then prepared for histology as described in 3.5.1.

Figure 4.4 shows that with H&E staining there were no structural differences in the infected or uninfected keratinocyte layers incubated at 33°C (Figure 4 a and b). In contrast, the infected stratified keratinocyte layers incubated at 37°C was disintegrated compared to the uninfected control incubated at the same temperature (Figure 4.4 c and d). Furthermore, in comparison, the infected keratinocyte layers incubated at 33°C was less affected than the infected layer incubated at 37°C (Figure 4.4 a compared to Figure 4.4 c). Although impairments are observed in the infected cell layers, multiple layers of keratinocytes are still seen in Figure 4.4.

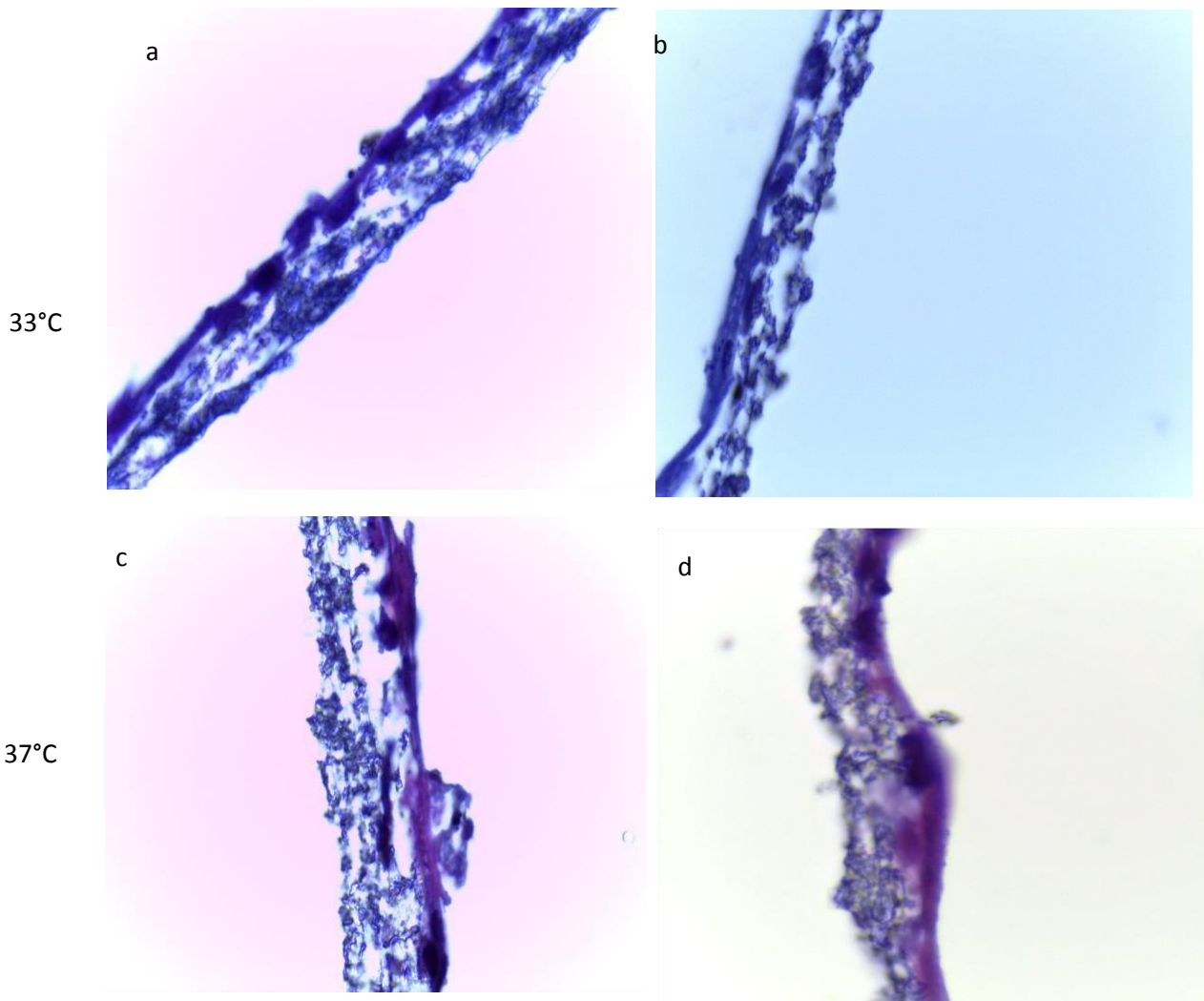


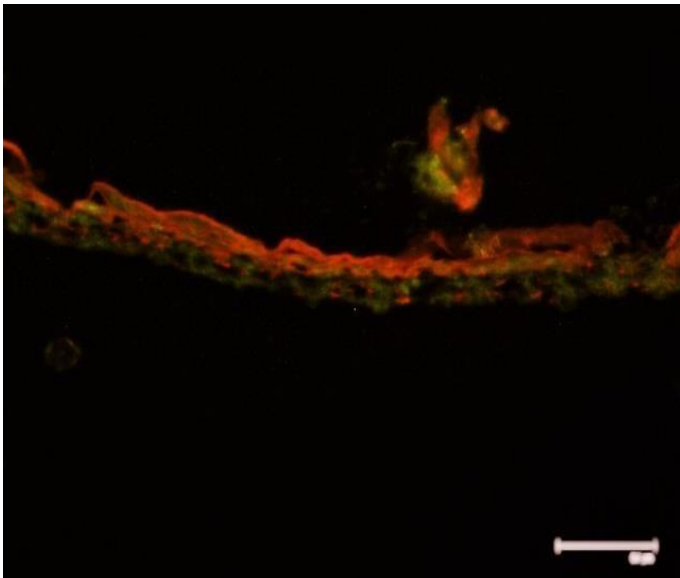
Figure 4.4: The comparison of the effect that *Chlamydia trachomatis* has on differentiated, polarised HaCaT cells at different temperatures.

Images from H&E staining shows cells that were incubated at 33°C (a) and 37°C (c). Uninfected HaCaT structures were also incubated at 33°C (b) and 37°C (d) X1000.

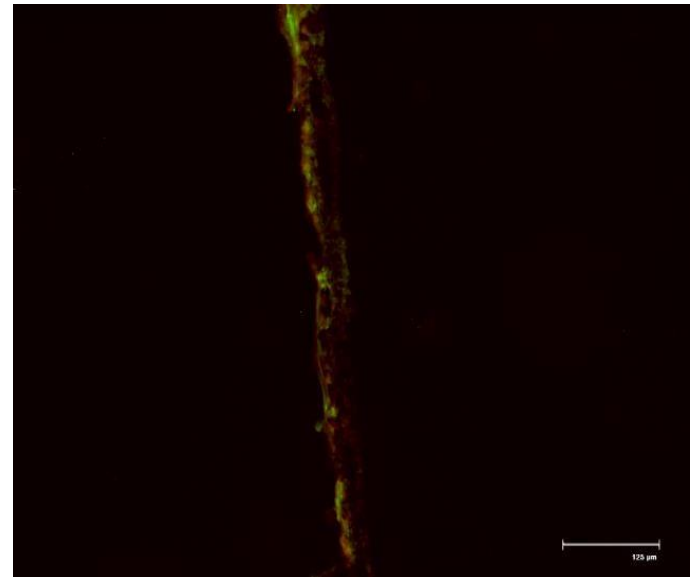
4.3 *C. trachomatis* infection of stratified HaCaT cells at 37°C and 33°C

In order to demonstrate the effect that *C. trachomatis* has on stratified keratinocyte layers at 33°C and 37°C, HaCaT cells grown at the air-liquid interface at the respective temperatures were infected with *C. trachomatis* and incubated for 7 days. The membrane inserts were then embedded in paraffin and sectioned (8 microns). In order to confirm infection with *C. trachomatis*, sections were stained with the Microtrak chlamydia trachomatis confirmation kit reagents. Sections were then viewed with the Invitrogen™ EVOS™ TL Cell imaging system.

Figure 4.5 shows infection was successful at both temperatures with cells containing *C. trachomatis* inclusions. Infected cells fluoresce green and uninfected cells red. There are less red cells in the keratinocyte layers incubated at 37°C as compared those incubated at 33°C, indicating that more cells were infected at 37°C. This is in keeping with the observation (4.2) that the keratinocyte layers incubated at 37°C are more disintegrated and broken than at 33°C.



33°C



37°C

Figure 4.5: *Chlamydia trachomatis* infected layered keratinocytes stained with the MicroTrak® *C. trachomatis* Culture Confirmation Test Kit reagents. Scale bar: 64µm (33°C); 125µm (37°C). Magnification: 70% (33°C); 50% (37°C).

4.4 Differentiation of chlamydia infected HaCaT cells

Membrane inserts with chlamydia infected keratinocyte layers as well as uninfected controls were processed as described in 3.5.1. The thin sections were stained with antibodies to keratin 10, keratin 14 and involucrin.

Figure 4.6 shows the presence of keratin 10, keratin 14 and involucrin. For keratin 10 and involucrin, fluorescence is more intense at 33°C than 37°C. For keratin 14, fluorescence is more intense at 37°C.

In addition, Figure 4.7 also shows that all sections from the structure of stratified keratinocytes incubated at 33°C were much more intact and unbroken compared to the brittle and disintegrated layers observed in the sections from structures incubated at 37°C, concurring with observations in Figures 4.4 and 4.5.

Figure 4.7 shows that non-specific binding of antibodies had not occurred after controls for nonspecific binding were prepared (3.5.3). White light images seen in Figure 4.7 b and d indicate the presence of the section, even though fluorescence was not detected.

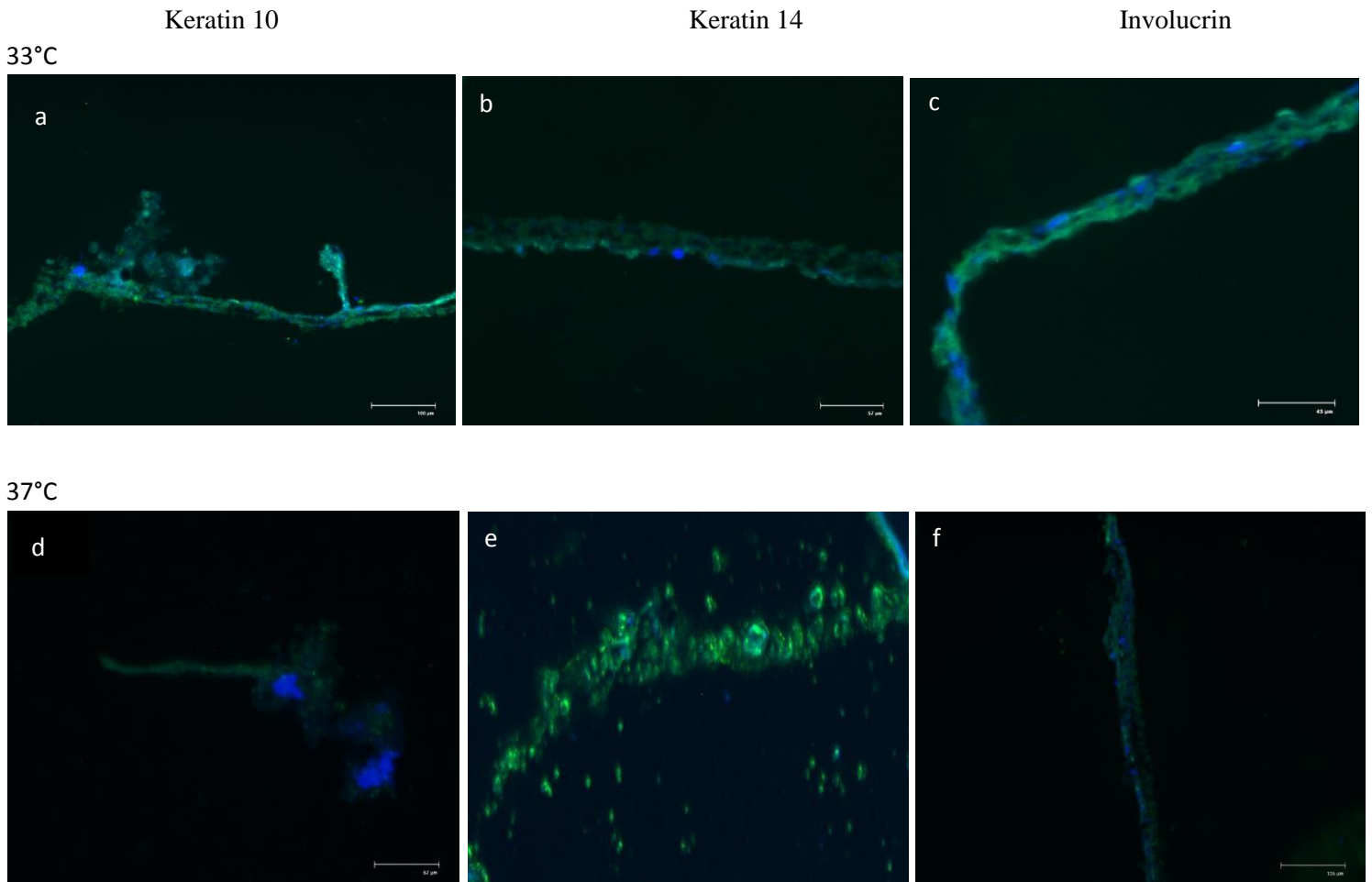


Figure 4.6: Confirmation of differentiation by the expression of keratinocyte markers (a-f).

Scale bar (a-f): 100μm; 57μm; 43μm; 67μm; 100μm; 125μm.

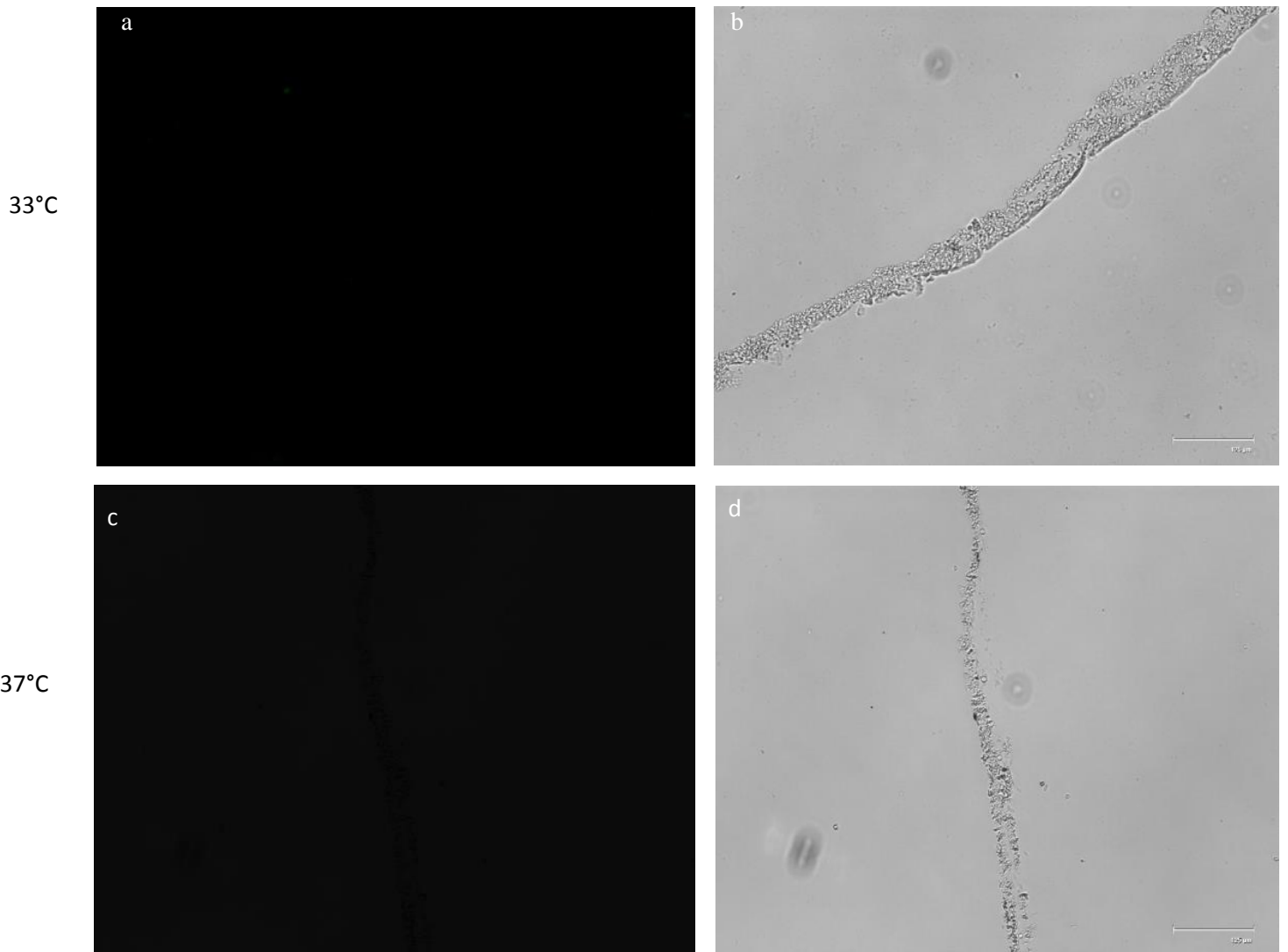


Figure 4.7: Control demonstrating that non-specific binding had not occurred (a-d). Scale bar: 125µm.

4.5 Controls for stratification of keratinocytes

In order to demonstrate the difference in the stratification and differentiation in keratinocytes after 7 days of incubation at either temperature (33°C and 37°C), as opposed to that after 24 days of incubation, HaCaT cells were grown on PTFE collagen inserts for 7 days in KGM-Gold media supplemented with

10% FBS and 1.4 mM calcium at 33°C and 37°C. Inserts were embedded in paraffin and sectioned. H&E staining was then performed on the sections

Figure 4.8 shows that only one solid and intact layer of keratinocytes was seen after 7 days of incubation at 33°C and 37°C, indicating that the keratinocytes had not stratified.

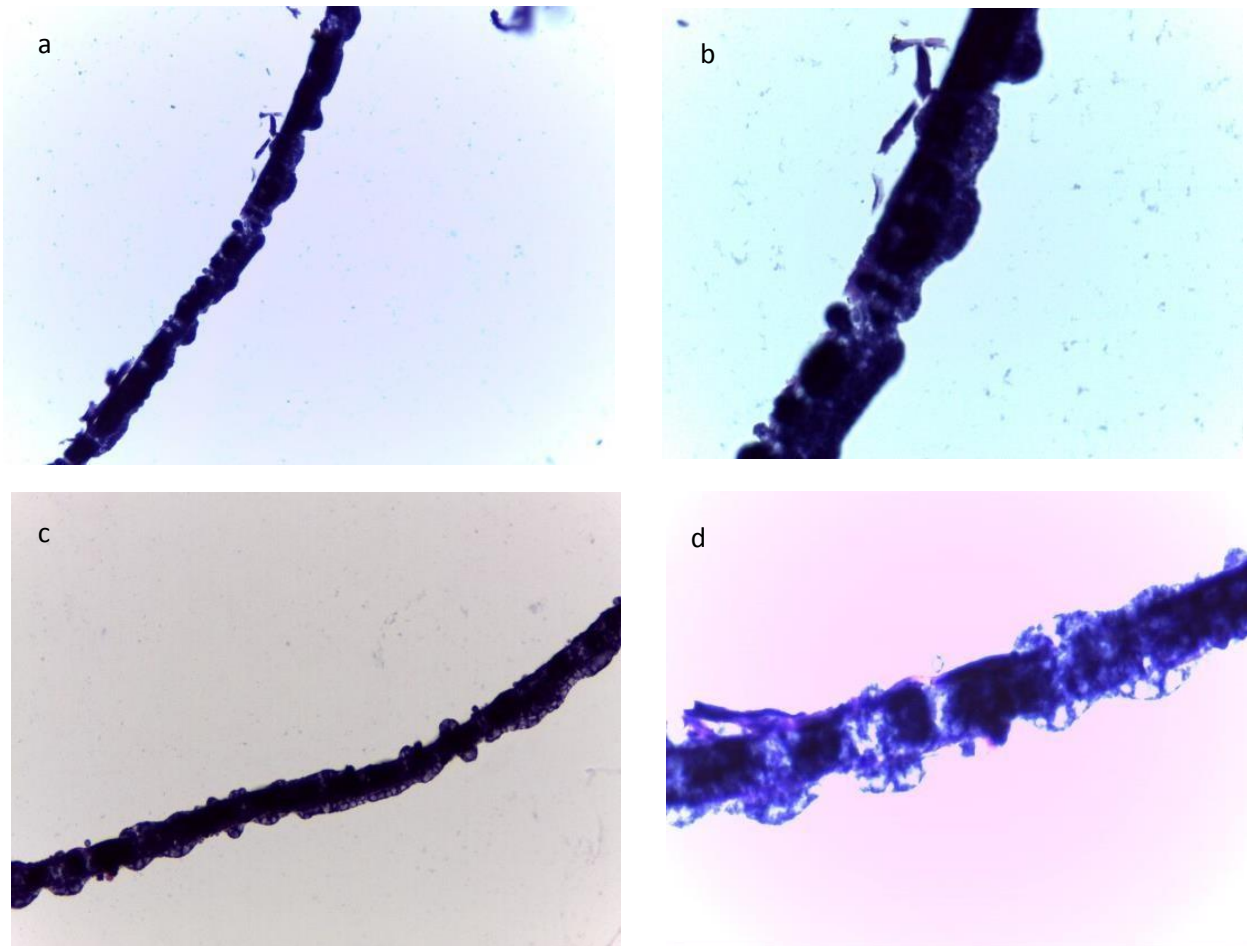


Figure 4.8: H& E stain of the control which demonstrates the staining pattern in non-polarised, non-differentiated keratinocytes incubated for 7 days at 33°C (a X400 and b X1000) and 37° (c X400 and d X1000).

CHAPTER 5 – DISCUSSION

Studies on stratification experiments have been limited mostly to primary human keratinocytes, which have been found to stratify and differentiate more easily and efficiently than keratinocytes cell lines (Lamb and Ambler, 2013).

Our study focused on generating *in vitro* stratified keratinocyte layers using HaCaT cells grown on collagen-coated PTFE Transwell® membrane inserts, instead of aiding stratification with additional cytokines and growth factors such as TGF- α and KGF. The aim was to use this to study the pathogenesis of *C. trachomatis*, since its LGV biovar has human skin as its primary target.

Methods to do so were first optimized for HaCaT cells. Stratification took place at both 33°C and 37°C. Although stratification was more prominent in keratinocyte layers incubated at 37°C, the expression of keratin 10 and involucrin were more intense in layers incubated at 33°C. *C. trachomatis* infection was successful in cells grown at both temperatures. However, more infected cells were seen at 37°C than at 33°C.

Marcello *et al* 1978, showed that after 3-4 days of incubation at 32°C - 33°C, a complete monolayer of Normal Epidermal Keratinocytes (NEKs) had formed and by 5-6 days, a second layer formed over the majority of the surface layer. These authors found that the upper layer constituted of cells with a

fibril network, allowing for a “basket weave” appearance of the keratinocytes. Stratification proceeded over 11 days in culture and after a total of 14 days, clumps of cells were visible. This primary keratinocyte model showed complete culture stratification, differentiation and proliferation after a 3 or more week incubation period. From this study (Marcello *et al*, 1978), it can be deduced that an incubation period of less than 3 weeks is not sufficient for stratification, differentiation and proliferation of keratinocytes. Our results with HaCaT cells shown in Figure 4.1 concur with these findings observed with NEK cells since a more intact HaCaT keratinocyte structure is seen after incubation for 24 days than after 14 days.

Breitkreutz *et al*, 1998, reported that the differentiation of HaCaT cells improved after approximately 21 days of incubation at 37°C, since typical features of differentiation, such as attachment structures and a basement membrane (BM) similar to NEKs were detected. Our study supports this finding (Fig 4.1).

Although more than one layer is seen in keratinocytes incubated for 14 days, thicker and clearer suprabasal layers are seen in keratinocytes incubated for 24 days. This indicates that HaCaT cells require three or more weeks to produce a differentiated and polarized structure comparable to human skin. It was for this reason that an incubation period of 24 days was used in all downstream experiments in this study, regardless of temperature.

When preparing the membranes for histology, the membranes were left inside the inserts since these are extremely delicate and needed to be handled carefully. Having the cells on the membrane whilst it is still in the insert, as opposed to using a loose membrane, prevents potential damage to the cells.

The manufacturer’s protocol for immunofluorescence staining called for heating of sections at 95°C (to facilitate antigen retrieval) in a microwave for two 5 minute intervals in a 10mM sodium citrate buffer, pH 3.5 with 0.01% (w/v) EDTA. However, there were no sections present on the slides after this step was conducted. To rule out the possibility of micro waving being too harsh, the sections were placed in a glass container with pre-heated 10mM sodium citrate buffer, pH 3.5 with 0.01% (w/v) EDTA. The container was then placed into a water bath at 95° for 10 minutes. This allowed for the sections to still be visible on the slides after heat-treatment.

A calcium gradient in the epidermis contributes towards the regulation of the process of differentiation in keratinocytes during their progression through various layers of the epidermis, eventually resulting in a permeable stratum corneum (Bikle *et al.* 2012). In our studies, the calcium concentration in the media was raised to 1.4mM in an effort to ensure that stratification and differentiation of HaCaT cells occurred. The KBM-Gold media, with which the cells were fed when exposed to the air-liquid interface, already had a calcium concentration of 0.1mM.

Although the exact role of calcium in keratinocyte differentiation remains unknown (Shrestha *et al.* 2016), it does affect signalling pathways such as the formation of desmosomes, adherens junctions and tight junctions (Bikle *et al.* 2012). In addition, the calcium receptor plays a pivotal role in promoting intracellular signalling processes which facilitate differentiation once extracellular calcium is detected (Bikle *et al.* 2012).

An increase in the extracellular calcium concentration allows for the internal release of, and an influx of calcium through the respective calcium channels in the plasma membrane (Lee *et al.* 1991). In turn, this facilitates cell to cell adhesion (Hennings *et al.* 1983) and increases the intracellular free calcium concentration, which regulates the differentiation of keratinocytes (Hennings *et al.* 1981).

Keratinocytes transcend from the stratum basale to the stratum corneum, and during that process they undergo terminal differentiation. This can be reproduced by growing keratinocytes in media that is enriched with a high calcium concentration (Henning *et al.* 1980). *In vitro* keratinocytes appear to look like basal epidermal cells and do not differentiate in media with a reduced calcium concentration (less than 0.03mM). However, at calcium concentrations over 0.1mM, keratinocytes resemble suprabasal epidermal cells and are seen to successfully differentiate (Hennings *et al.* 1981).

A study by Steven *et al.* 1983, which investigated the regulation of differentiation of NEKs by calcium, demonstrated that a calcium concentration of 0.3mM in the media, allowed for a colonyforming efficiency of 30% and a cellular multiplication rate of 0.96 doublings per day. An increased calcium concentration (0.1mM) allowed for stratification and terminal differentiation of the NEKs and this was determined by counting the number of cornified envelopes that remained after boiling in sodium dodecyl sulphate with dithithreitol.

Similarly, another study by Hennings *et al.* 1980, investigated the role of calcium in the differentiation of cultured mouse keratinocytes. In an environment where calcium in the medium was approximately 0.05mM-0.1mM, increased proliferation of the keratinocytes and production of keratin took place, albeit no stratification was seen. Upon ultrastructural examination after cells were subcultured in a reduced calcium environment, a vast majority of intracellular spaces was seen. The initiation of terminal differentiation was then conducted by increasing the concentration of calcium in the media to 1.2mM, thereby substantially improving cell to cell contact and the formation of desmosomes in only 2 hours. After 2 days, stratification of the cells was observed and terminal differentiation, indicated by cell sloughing, took place after 3 days.

More recently, a study by Lamb and Ambler, 2013, investigated the stratification displayed by keratinocytes in various media types with varying concentrations of calcium. Hematoxylin and eosin (H&E) staining showed that low calcium medium (0.05mM), supplemented with 10% serum did not result in the differentiation and stratification of NEKs. Contrastingly, cells grown in an environment with increased calcium (1.4mM) allowed for the production of two clearly visible layers of nucleated cells, indicating that an increased calcium environment is essential for the stratification and differentiation of keratinocytes.

In order to examine an alternate method of developing stratified keratinocyte layers with HaCaT cells, organotypic co-cultures of HaCaT cells and fibroblasts were grown on a collagen bed.

Stromal interactions, initiated by IL-1 are required for differentiation and this cytokine is present in NEKs but reduced in HaCaT cells (Maas-Szabowski *et al.*, 2003). These interactions allow for the subsequent production of various growth factors such as keratinocyte growth factor (KGF/FGF-7) and granulocyte macrophage-colony stimulating factor (GM-CSF) in fibroblasts (Maas-Szabowski *et al.*, 2001). These factors in turn stimulate growth and differentiation of keratinocytes. The expression of the receptors for these growth factors in HaCaT cells is substantially reduced, suggesting that simply adding KGF and GM-CSF to the media, will not be sufficient to promote stratification and differentiation of the HaCaT cells (Maas-Szabowski *et al.*, 2003). In addition, the release of the keratinocyte growth factor TGF- α is also reduced in HaCaT cells (Maas-Szabowski *et al.*, 2003).

Maas-Szabowski *et al.*, 2003, investigated the stratification and differentiation of HaCaT cells grown as part of organotypic co-cultures with fibroblasts, with the addition of various growth factors and compared it to that seen in NEKs. The study found that co-cultures supplemented with TGF β and IL-1 allowed for the increased expression of KGF and GM-CSF, thereby producing a differentiated and stratified model of HaCaT cells comparable to both, NEKS and human *in vivo* skin.

We used the same method for growing organotypic co-cultures of HaCaT cells and fibroblasts in order to examine an alternate method of generating an *in vitro* model of stratified keratinocytes that closely resembled *in vivo* skin as opposed to only growing HaCaT cells at the air-liquid interface in PTFE-collagen coated membrane inserts. However, the outcome of our attempt differed from the results seen by Maas-Szabowski *et al.*, 2003 in that the collagen had shrunk after being incubated with fibroblast-HaCaT co-cultures at both 33°C and 37°C (Figure 4.3).

A study by Li and Wang, 2011, investigated the role of fibroblasts and myofibroblasts in wound healing. Cell traction forces allow fibroblasts and myofibroblasts to move throughout the extracellular

matrix (ECM) (Harris *et al.* 1981) and disrupt the ECM network, resulting in the regulation of cellular functions (Tranquillo *et al.* 1992), maintaining homeostasis with regards to cellular tension (Harris *et al.* 1987). In comparison, fibroblasts produce traction forces larger than those required for cellular movement (Harris *et al.* 1981) causing major disruptions of the cell ECM. More closely, during tissue repair, fibroblasts produced traction forces that caused contraction and disruption of the collagen matrix. In addition, a study by Elrich *et al.* 1990 showed that fibroblasts produced a traction force that caused single units to contract the wound and that myofibroblasts indicated the conclusion of the production of the contractile force.

Taking the aforementioned studies into consideration, it may be assumed that the shrinkage of collagen observed in Figure 4.3 may have been as a result of the contractile forces of fibroblasts similar to what happens during wound healing. Since the collagen layer had shrunk to a much smaller size when incubated at 37°C (Figure 4.3 b), compared 33°C (Figure 4.3 a), it may be assumed that 37°C is the optimum temperature for the cellular processes of fibroblasts, allowing for an increased production of the contractile force.

In order to potentially generate a stratified keratinocyte structure using a method that involves growing organotypic co-cultures on a collagen bed, the amount of collagen used in the experiment would have to be optimized in order to counteract the amount of surface area that will be lost due to shrinkage. This method was not investigated further. However, it leaves room for additional studies.

The *in vitro* keratinocyte layers were incubated at different temperatures to determine which of the two allowed for a closer resemblance to human skin *in vivo*. The two temperatures that were chosen for this experiment were 33°C and 37°C. This is because 37°C is core body temperature and 33°C is the temperature of human skin (Ramanathan, 1964).

All studies involving the development of a stratified keratinocyte model have used incubation at 37°C. Therefore, a model where HaCaT cells are grown at 33°C has not been studied before. In keeping with Lamb and Ambler, 2013, we detected the presence of keratin 10, keratin 14 and involucrin in cells grown at 37°C. Keratin 10, keratin 14 and involucrin were also detected in the cells that had been incubated at 33°C (Figure 4.6). This indicates that the keratinocytes in the stratification models incubated at both temperatures had committed to differentiation, although the keratin 10 and involucrin fluorescence was more intense at 33°C than at 37°C, whilst the fluorescence of keratin 14 remained more intense at 37°C.

As keratinocytes differentiate, there is detectable down-regulation of a specific set of basal proteins and the up-regulation of suprabasal markers (Fuchs, 1990). Keratins are expressed by epithelial tissue at specific sites and at specific stages of differentiation (Schweizer *et al.* 2006). It is for this reason that we looked for the presence of the basal marker keratin 14 and suprabasal markers keratin 10 and involucrin.

Keratin 14 is part of the type I keratin family of intermediate filaments and is therefore involved in forming the cytoskeleton of epithelial cells and is expressed in mitotically active basal layer cells simultaneously with keratin 5 (Schweizer *et al.* 2006). As cells differentiate, expression is down regulated (Schweizer *et al.*, 2006). Keratin 10, on the other hand, is expressed in post-mitotic, terminally differentiating keratinocytes (Paramio *et al.* 1999). During terminal differentiation, the cornified envelope develops in keratinocytes, causing cells to become permeable to both, calcium and various other ions (Thacher & Rice, 1985). This influx of calcium allows for the activation of transglutaminase which catalyses this process. (Thacher & Rice, 1985).

Involucrin is an imperative precursor of the cell envelope. The mechanism by which Involucrin is positioned remains unknown, however it is placed adjacent to the inner plasma membrane and

crosslinked to a number of membrane proteins by keratin-specific transglutaminase (Thacher & Rice, 1985). The sub-cellular localization of precursors and the involucrin amino acid sequence allows for the generation of the envelope and this process involves a minimum of 6 proteins (Thacher & Rice, 1985).

Figure 4.6 also shows that a more intense fluorescence is seen at 33°C for keratin 10 and involucrin compared to that seen at 37°C. This indicates a higher concentration of suprabasal markers, suggesting that even though stratification of keratinocytes was slower at 33°C than 37°C, differentiation was quicker at 33°C. Gene expression studies on these two markers at different temperatures should be done in order to substantiate this finding.

Figures 4.4 b and d show that more layers are formed during incubation at 37°C compared to 33°C, indicating that 37°C is a more suitable temperature to grow stratified keratinocyte layers. This may be because 37°C is the core body temperature (Ramanathan, 1964), giving an indication of how keratinocytes may grow in the human body.

Additionally, further studies should be conducted where keratinocytes are grown at a temperature range from 30° to 37°C to better understand how *in vivo* keratinocyte growth does take place.

After infection of the keratinocytes with chlamydia, the cells were incubated for both the experiments at 33°C and 37°C for a further week. The life cycle of the *C. trachomatis* L2 strain is approximately 48 hours (Choroszy-krol *et al.* 2012). Therefore, incubating the infected keratinocyte layers for a week after infection ensured that EBs were released after the first cycle to infect new cells within the keratinocyte structure, allowing deeper penetration of the microorganisms into the keratinocyte layers.

C. trachomatis infection experiments of multiple layers of HaCaT cells grown at 33°C have not been conducted before. Joubert and Sturm, 2008 showed that a monolayer of HaCaT cells grown at 33°C

could be infected with *C. trachomatis*, which is the approximate temperature at the initial target site during infection with the LGV biovar. The results of this study (Figure 4.5) show that *C. trachomatis* also infects layered keratinocytes at 33°C.

Degradation seen in the infected keratinocyte layers incubated at 37°C was much more prominent compared to that incubated at 33°C (Fig 4.4 and 4.5). The layers formed at 33°C were not largely infected with *C. trachomatis* since there was a substantial amount of uninfected cells present at 33°C as compared to 37°C (Fig. 4.5). This is in keeping with former findings that the optimum growth temperature of the *C. trachomatis* LGV biovar is 37°C (Joubert and Sturm, 2008).

Overall, the study provided an idea of the pathology of the *C. trachomatis* by demonstrating the effect it may have on *in vivo* skin. Similar keratinocyte models may be generated and infected with various other organisms in an effort to gain insight on the pathology thereof. In addition, the study may be explored further by documenting the effect of the organism on keratinocytes at various stages of the duration of incubation, giving a more detailed analysis of its pathology.

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APPENDIX A

Media, Solutions and Reagents

1. Cell Culture Media

1.1. Chlamydia Growth Media for Propagation

20 ml EMEM with NEAA

1 ml EMEM 20X glucose (1.76g glucose in 20ml EMEM)

0.02 ml Gentamycin

0.02 ml Amphotericin B (10mg/ml)

0.02 ml Cycloheximide (1mg/ml)

Reagents were added and refrigerated until use

1.2. All other cell culture media were used as purchased and respective supplements were added when necessary, as highlighted in Chapter 3

2. Phosphate Buffered Saline (PBS) (Dulbecco A)

5 PBS tablets (Oxoid)

500ml distilled water

After dissolving 5 PBS tablets in 500ml autoclaved distilled water, the mixture was autoclaved at 121°C for 10 minutes, cooled and 20 ml aliquots were made in plastic tubes for refrigeration until use.

3. 0.05% EDTA

0.05g EDTA

1 PBS Tablet

100 ml Distilled water

After dissolving 1 PBS tablet and 0.05g EDTA in ~80ml autoclaved water, the pH was adjusted to 7.4 by adding 7.5% Sodium bicarbonate. Thereafter, the volume was adjusted to 100 ml and the mixture was autoclaved at 121°C for 10 minutes. 1 ml aliquots were then prepared for refrigeration until use.

4. Storage Fluid

4.1. HaCaT Cell Storage Fluid

30 ml Hyclone DMEM

10 ml Glycerol (BDH Laboratory supplies, Poole, England)

10 ml FBS

10 ml FBS and 10 ml Glycerol were added to 30 ml Hyclone DMEM and filter sterilized through a 0.22µm filter into a sterile 50ml tube.

4.2. McCoy Cell Storage Fluid

30 ml EMEM with EBSS, NEAA, sodium pyruvate, L-glutamine and HEPES

10 ml dimethyl sulphoxide (DMSO) (Fluka, Steinheim, Germany)

10 ml FBS

10 ml DMSO and 10ml of FBS were added to 30ml of EMEM and filter-sterilized through a 0.22µm filter into a sterile tube.

5. MicroTrak® *C. trachomatis* Reagent

5.1. MicroTrak® *C. trachomatis* Culture Confirmation Test:

C. trachomatis reagent: Fluorescein-labelled mouse monoclonal antibodies specified to *C. trachomatis*; protein-stabilized phosphate buffer; Evans Blue counter-stain, 0.01% sodium azide

Diluent: deionized water; 0.1% sodium azide

3 ml reconstitution diluent was added to lyophilized *C. trachomatis* reagent and gently swirled in order to dissolve. The reconstitution date was recorded on the vial, which was allowed to reach room temperature after approximately 15 minutes. Thereafter, the vial could be refrigerated (2-8°C) for up to 12 weeks.

5.2. Sucrose-Phosphate-Glutamate Buffer (SPG)

Sucrose

KH₂PO₄

Na₂HPO₄. 7H₂O

Glutamic Acid

Distilled water

The Sucrose, KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and Glutamic Acid were dissolved in ~400 ml autoclaved distilled water and the pH was adjusted to 7.4 by adding 7.5% Sodium bicarbonate. Thereafter, the volume was brought to 500 ml and the solution was filter sterilized through a 0.22 μm filter into a sterile tube and stored at -20°C until use.

6. Immunofluorescence

6.1. Primary Antibodies

Antibodies to keratin 10, keratin 14 and involucrin were diluted in PBS and 1.5% Goat serum as follows:

1:50 – 20 μl antibody was added to 980 μl PBS + Goat Serum Solution

1:500 – 2 μl antibody was added to 998 μl PBS + Goat Serum Solution

6.2. Secondary Antibodies

Secondary antibody conjugated to FITC were diluted in PBS and 1.5% Goat serum as follows:

1:100 – 10 μl antibody was added to 990 μl PBS + Goat Serum Solution

1:400 – 2.5 μl antibody was added to 997.5 μl PBS + Goat Serum Solution

6.3. 1.5% Goat Serum

0.15 ml Goat Serum

9.85 ml PBS

0.15 ml Goat serum was added to 9.85 ml PBS and refrigerated until use

6.4. Sodium Citrate Buffer

2.94g Tri Sodium Citrate (dihydrate)

1000ml distilled water

2.94g Tri Sodium Citrate (dehydrate) was dissolved to 1000 ml distilled water and the pH was adjusted to 6.0 by adding hydrochloric acid

6.5. Blocking Serum

0.5 ml Goat serum

4.5 ml PBS

0.5 ml Goat serum was added to 4.5 ml PBS and refrigerated until use

7. Preparation of collagen and organotypic co-cultures

7.1. 5 mg Collagen from rat's tail (Sigma)

0.1% acetic acid

0.15 ml 10X HBSS

5 mg collagen was dissolved in 0.1% acetic acid on ice. The solution was then added to 0.15 ml 10X HBSS and neutralized with 2M NaOH

7.2. Mitomycin

120 mg Mitomycin was dissolved in 30 ml autoclaved distilled water and aliquotted into 1 ml tubes and refrigerated for use