Human Organotypic Models for Anti-infective Research

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Abstract The use of human organotypic models for biomedical research is experiencing a significant increase due to their biological relevance, the possibility to perform high-throughput analyses, and their cost efficiency. In the field of anti-infective research, comprising the search for novel antipathogenic treatments including vaccines, efforts have been made to reduce the use of animal models. That is due to two main reasons: unreliability of data obtained with animal models and

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the increasing willingness to reduce the use of animals in research for ethical reasons. Human three-dimensional (3-D) models may substitute and/or complement in vivo studies, to increase the translational value of preclinical data. Here, we provide an overview of recent studies utilizing human organotypic models, resembling features of the cervix, intestine, lungs, brain, and skin in the context of anti-infective research. Furthermore, we focus on the future applications of human skin models and present methodological protocols to culture human skin equivalents and human skin explants.

1 Introduction

Overall mortality caused by infectious diseases has dramatically decreased in the past decades due to the positive impact of antibiotics, antivirals, antifungals, vaccines, and more recently monoclonal antibodies. The use of in vitro and/or in vivo models in preclinical research aims to predict the level of safety, immunogenicity, and protection of novel anti-infective treatments. Unfortunately, animal models often fail in this respect, as shown by the high number of unsuccessful clinical trials. There is an abundant amount of pathogens with exclusive human-specific properties, thus underlining the need for human in vitro or ex vivo models to study these microbes and to obtain reliable results in preclinical studies. Furthermore, great efforts are being made to reduce, refine, and replace the use of animal models in various fields of biomedical research. Human organotypic models are becoming more popular in both the academic sector as well as industry, producing valuable information to help find novel therapeutic targets. Here, we will present an overview of human organ-specific models used in anti-infective research, focusing on cervical, intestinal, respiratory, brain, and skin models. Moreover, we will dedicate a section of this review to human skin models commenting on their future applications and presenting a methodological protocol for the culture of human skin explants and skin equivalents that can be applied to anti-infective research.

2 Part 1—State of the Art of Organotypic Models for Anti-infective Research

2.1 Cervical Models

Human 3-D organotypic cultures, also known as raft cultures, have been developed in order to mimic human cervical epithelium. These models are frequently composed of primary cervical keratinocytes or cervical carcinoma cell lines seeded on top of a collagen matrix filled with fibroblasts (Delvenne et al. 2001). Organotypic raft cultures are then grown at air–liquid interface in order to promote differentiation and consequent stratification of the epithelium.
Epithelial differentiation is essential for human papillomavirus (HPV) replication (Meyers et al. 1992); thus, cell monolayers are limited models to study the life cycle of HPV and, consequently, to develop antiviral therapies against its replication. For this reason, organotypic epithelial raft cultures have been used to evaluate the efficacy of antivirals in blocking replication of different HPV types. Two commercial preparations of interferon-α (IFN-α) (Sen et al. 2005) and an antisense oligonucleotide targeting the mRNA of an essential protein (ORI-1001) for HPV6 and HPV11 replication (Alam et al. 2005) were tested in this model. These antivirals were able to impair the replication of specific HPV types, and the optimal concentration of the two IFN-α preparations was shown to be different for each of the HPV types. Additionally, the efficacy of bi-specific monoclonal antibodies anti-CD3/anti-EGFR, designed to target epidermal growth factor receptor (EGFR)-positive neoplastic keratinocytes by activating T cells (Ferrini et al. 1993), was also evaluated in a human 3-D model of the cervical epithelium (Renard et al. 2002). After the demonstration that lymphocytes can penetrate in 3-D cultures composed by a cervical carcinoma cell line (Jacobs et al. 1998), the authors proved that T cells, previously incubated with the bi-specific antibodies, induce apoptosis of HPV16-transformed primary keratinocytes, but not of healthy keratinocytes (Renard et al. 2002). These studies highlighted the feasibility and preclinical relevance of organotypic cultures of keratinocytes to test different classes of anti-HPV agents.

Human cervical explants have also been extensively used to screen the efficacy and toxicity of topical antiviral candidates, mainly for human immunodeficiency virus (HIV). The compounds tested include polyanionic molecules, such as the synthetic naphthalenesulfonate polymer PRO2000 and dextrin sulfate (DxS); cellulose acetate 1,2-benzenedicarboxylate (CAP); the carrageenan-based product PC-515; the lysine dendrimer SPL7013; the antimicrobial peptide D2A21; cyanovirin-N; palmitic acid; nonoxynol-9; gramicidin; the pyrimidinedione IQP-0528; and the non-nucleoside reverse transcriptase inhibitor UC781 (Greenhead et al. 2000; Zussman et al. 2003; Fletcher et al. 2005, 2006; Lu et al. 2006; Cummins et al. 2007; Buffa et al. 2009; Lin et al. 2011; Mahalingam et al. 2011). The combinatorial effect of these compounds in preventing HIV-1 infection has also been evaluated in polarized ectocervical explants (Zhang et al. 2015).

Infections caused by HIV are frequently accompanied by other sexually transmitted infections, such as herpes simplex virus (HSV) (Wald and Link 2002; Buve 2010). Highly specific drugs against each of these viruses have been designed, including acyclovir (HSV-specific) and tenofovir (HIV-specific). Surprisingly, clinical trials revealed that acyclovir could reduce HIV-1 levels in HSV-2/HIV-1 coinfected patients (Nagot et al. 2007; Zuckerman et al. 2007; Baeten et al. 2008) and that tenofovir reduced the risk of HSV-2 infection (Cates 2010). The ability of acyclovir to suppress HIV-1 infection was confirmed in human tonsils, lymph nodes, cervicovaginal and colorectal tissues inoculated with HIV-1 and HSV-2 (Lisco et al. 2008), and the effectiveness of tenofovir to impair HSV replication was evaluated in several in vitro and ex vivo models such as organotypic epithelial 3-D rafts, human lymphoid and cervical tissues (Andrei et al. 2011). Therefore, cervical and other types of tissues showed to be suitable models to assess the antiviral effect.
of microbicides against two different viruses. In a more recent study, the compound PMEO-DAPym (6-phosphonylmethoxyethoxy-2,4-diaminopyrimidine) was also identified to target both HSV and HIV infections (Balzarini et al. 2013). Its potency was also tested in multiple models, including organotypic epithelial raft cultures of human primary keratinocytes and human lymphoid and cervical tissues, showing an overall significant advantage over the existing compounds tenofovir and adefovir, which have been described to impair both HIV and HSV.

In summary, human cervical raft cultures and explants have been exploited as models to test the efficacy of various treatments, to optimize the concentration of microbicides and to assess the benefit of combining antivirals to tackle different viruses. Importantly, organotypic models have been used to overcome the limitations of cell monolayers in reproducing in vivo conditions essential for viral replication.

2.2 Intestinal Models

In the last years, several studies started to use colorectal explant tissues to evaluate the antiretroviral efficacy of topical microbicides marketed or under evaluation in clinical trials for vaginal use. The safety and effectiveness of many antivirals such as CAP, PRO2000, SPL7013, Vena Gel, and the class of reverse transcriptase inhibitors (RTI), which includes the nucleotide-RTI tenofovir, the nucleoside-RTI emtricitabine (FTC) and the non-nucleoside-RTI UC-781, dapivirine (TMC120), and IQP-0528 was assessed in colorectal tissues (Abner et al. 2005; Fletcher et al. 2006; Herrera et al. 2009; Rohan et al. 2010; Herrera et al. 2011; Dezzutti et al. 2012, 2014). When tested in combination, RTI were shown to induce a greater inhibitory potential compared with the compounds tested alone (Herrera et al. 2009, 2011). The effectiveness of other types of drugs, such as the polyanion candidates PRO2000 and DxS, has been also tested in parallel with the RTI activity (Fletcher et al. 2006). More recently, a study combining the non-nucleoside-RTI TMC120 with the HIV entry inhibitor maraviroc proved a synergistic antiretroviral effect in both ectocervical and colonic tissue (Dezzutti et al. 2015). Also, in this case, the use of explant tissue models allows the preclinical assessment of both toxicity and efficacy of topical microbicides, and it facilitates the screening of multiple candidates, alone or in combination, against different viral subtypes, thereby being less laborious than in vivo models.

Another type of human intestinal organotypic model is the 3-D cultured primary intestinal organoid, which is an organ-like tissue generated from pluripotent stem cells isolated from intestinal tissue (Lancaster and Knoblich 2014). Organoids contain various types of cells and recapitulate most aspects of in vivo tissue architecture, like spatial organization and overall microenvironment. Intestinal organoids have been used to study host-pathogen interactions; nevertheless, the number of studies that uses this model to evaluate the efficacy of antimicrobial agents is still limited. Yin et al. showed that primary human intestinal organoids were permissive to rotavirus infection, both to laboratory and patient-derived
rotavirus strains. Importantly, clinical isolates demonstrated different sensitivities to the antivirals IFN-α and ribavirin, suggesting that this organotypic model could be valuable to predict the individual responsiveness to antiviral therapy, thus supporting personalized medicine (Yin et al. 2015). Another study implemented a functional assay to determine the efficacy of cholera toxin inhibitors by measuring the swelling of primary human intestinal organoids (Zomer-van Ommen, Pukin et al. 2016).

2.3 Respiratory Models

The respiratory system has a complex architecture and consists of different distinct structures such as the nasal mucosa, the upper and lower bronchial tracts and alveoli. Due to the direct contact with the air, natural exposure to various pathogens is very common. Subsequently, pneumonia remains one of the leading causes of death worldwide, thereby making the respiratory system a key target model for anti-infective research (Global Burden of Disease Study 2015). Certain aspects of the respiratory system, such as the structural complexity and the different cell types involved, make it difficult to fully simulate all features in vitro. Different 3-D models, which mimic specific components or processes of the respiratory system, have been extensively used to test various novel anti-infective agents. For example, the protective role of nasal sprays in maintaining the barrier function of nasal epithelial cells has been shown in vitro using a commercially available 3-D nasal mucosal model (De Servi et al. 2017). The ability of a novel antimicrobial peptide, derived from frog skin, to affect the permeability of lung epithelium has been shown using primary bronchial epithelial cells differentiated in vitro, where the barrier integrity was measured with an electrode determining the transepithelial electrical resistance (TEER) (Chen et al. 2017). A 3-D co-culture system consisting of the human airway epithelial cell line BEAS-2B, immature dendritic cells (DCs) derived from human circulating CD14+ monocytes and human lung fibroblast cell line MRC-5, was recently used to discriminate skin sensitizers from respiratory sensitizers. In this model, OX40L upregulation in DCs was preferentially induced by respiratory sensitizers and was found to be associated with the initiation of the T helper type 2 (Th2) cell response leading to respiratory allergy (Mizoguchi et al. 2017). Similarly, a 3-D lung model, including an epithelial cell line in combination with a human lung fibroblast cell line, was used to test the effect of pooled immunoglobulin (IVIG) in preventing S. aureus toxins mediated tissue damage. In addition, live imaging analysis of GFP-expressing epithelial cells within these tissue cultures confirmed the protective effect of IVIG (Mairpady Shambat et al. 2015).

Ex vivo studies of lung-related samples are proving useful in anti-infective research as well. For example, the benefit of the vaccine adjuvant CpG to promote T follicular helper cells, which are important in eliciting a robust anti-influenza antibody response, has been shown in nasopharynx-associated lymphoid tissues.
(NALT) derived from healthy children and adults (Aljurayyan et al. 2016). Furthermore, analysis of *Pseudomonas aeruginosa* biofilm aggregates in sputum from cystic fibrosis patients, treated with low concentrations of nitric oxide (NO), showed an enhanced efficacy of antibiotics in treating chronic infection (Howlin et al. 2017). Although beyond the scope of this review, it should be mentioned that numerous in vitro and ex vivo 3-D models are widely being used to study infection of bacteria as well as viruses and fungi and could be important models to test novel anti-infective treatments in the future. In brief, ex vivo models such as human nasal respiratory mucosa have been used in the context of viral infection for studying herpes simplex virus type 1 invasion (Glorieux et al. 2011; Wang et al. 2012). Besides this, infections with *Mycobacterium tuberculosis*, *Aspergillus fumigatus*, and *Burkholderia cenocepacia* have also been extensively studied (Chandorkar et al. 2017; Fonseca et al. 2017; Van den Driessche et al. 2017). A future potential application of these models can be to test the efficacy of nasal nanovaccines, which represent a promising novel anti-infective (Bernocchi et al. 2017). In conclusion, 3-D organotypic human lung cell culture models can provide important information on the efficacy of novel antimicrobials.

### 2.4 Brain Models

Due to the large anatomical differences between the human brain and other mammals, there is a great need to establish experimental human brain models. However, since the brain contains a dense network of various cell types such as neurons, glial cells, and astrocytes, with complex interaction patterns, it is very challenging to recapitulate this system in vitro. Over the past years, a lot of effort has been made to this cause, e.g., in trying to develop neurospheroids and cerebral organoids (Jorfi et al. 2017). Many studies utilized 3-D human brain models to study the pathogenesis and identify potential therapeutic targets of infectious agents causing damage to the brain. A major advantage of using these models is that they allow for the high-throughput screening of potential anti-infectives, a very important feature in the response to the fast emergence of Zika virus. A pool of existing drugs has been tested for efficacy against Zika and Japanese encephalitis virus infection in in vitro models including fetal-like organoids, human fetal-derived neuronal stem cells, human cortical organoids, and human pluripotent stem cell-derived cortical neural progenitor cells (hNPCs) (Xu et al. 2016; Bullard-Feibelman et al. 2017; Li et al. 2017; Wang et al. 2017; Zhou et al. 2017). Here, it should be noted that the in vitro efficacy of antivirals has been confirmed by additional in vivo experiments in these studies. However, in the case of human-specific pathogens such as varicella zoster virus (VZV), the use of 3-D human brain models is even more critical. Human neuronal systems, derived from embryonic and neural stem cells, have been established to study the various stages of VZV infection and might function as a tool to investigate novel antiviral targets (Markus et al. 2015; Kurapati et al. 2017). Another study aimed to mimic the
human blood–brain barrier (BBB) by culturing a human brain microvascular endothelial cell line (HBMEC) in a rotating wall vessel (RWV) bioreactor. This model can be used to study the invasion of pathogens into the central nervous system (Bramley et al. 2017). This system may enable the screening of novel or existing antivirals for their protective effect. Collectively, human brain models are extensively being used as a high-throughput screening tool for anti-infective research, in particular for antivirals. However, data from currently used 3-D models still require additional studies in animal models, due to the very complex characteristics of the human brain.

3 Part 2—in-Depth Overview of Skin Models for Anti-infective Research

The skin is the largest human organ and functions as a barrier against physical injury, infections, and environmental cues (e.g., UV light) and contributes to body temperature regulation (Proksch et al. 2008; Olaniyi et al. 2017). The skin is composed of two major layers: the epidermis and the dermis. Keratinocytes are the main cellular component of the epidermis and its stage of differentiation subdivide the epidermis into four strata: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The outermost layer, stratum corneum, is populated by terminally differentiated keratinocytes, while stratum basale is the inner layer of the epidermis, which is composed of undifferentiated keratinocytes. Although less abundant, Langerhans cells, melanocytes, and cytotoxic T cells can also be found in the epidermis. The dermis is mainly composed of collagen and elastin fibers and is rich in immune cells such as dendritic cells, T cells, macrophages, and mast cells. Fibroblasts, nerves, as well as blood and lymphatic vessels are also present in this layer. In addition, epidermal appendages, such as sweat and sebaceous glands as well as hair follicles, are embedded in the dermal layer. Hair follicles transverse the two compartments of the skin, representing a major route of penetration for everything coming into contact with the skin, including microbes.

Keratinocytes are the first players to act when the skin barrier is challenged. Therefore, 2-D monolayers of keratinocytes have been used to study the primary immune response induced by keratinocytes in multiple fields, ranging from cosmetics to injury and pathogen infection. Nevertheless, monolayers of keratinocytes do not resemble the stratification characteristic of the epidermis. In order to overcome this limitation, reconstructed human epidermis (RHE) models can be built by culturing keratinocytes at the air–liquid interface (Netzlaff et al. 2005).

However, a complete model of the skin is needed to study host–pathogen interactions and anti-infective interventions, including their toxicity, efficacy, and induced immune response. Since animal experimentation is time-consuming, expensive, and its predictive value is limited when applied to humans, the ideal model should reproduce both histology and architecture of the human skin.
Full-thickness skin models, such as human skin equivalents and human skin explants, fulfill these requirements.

### 3.1 Skin Equivalent Versus Skin Explants

Many studies describe the development of skin equivalent models where the different layers of the skin are rebuilt using primary fibroblasts within a matrix of collagen followed by seeding with keratinocytes. Further differentiation of keratinocytes at air–liquid interface results in stratified layers, closely resembling the human skin. De-vitalized de-epidermalized dermis (DED), obtained by discarding both epidermis and adipose tissue from human skin, can also be used as a more physiological support to grow keratinocytes (Lamb and Ambler 2014; Ventress et al. 2016). In addition, either primary or immortalized keratinocytes can be used to construct the epidermal layer, although the widely used HaCaT cell line does not reach the terminal differentiation stage (Boelsma et al. 1999; Schoop et al. 1999). Human skin explants, commonly collected from cosmetic surgery procedures, represent the most complete skin model.

When selecting a model to evaluate the efficacy or toxicity of an anti-infective compound, it should be taken into account that each of the full-thickness human skin models, which include skin equivalents and skin explants, presents advantages and disadvantages (Table 1).

In order to compensate for the lack of immune cells in the skin equivalent models, Langerhans cells (Dezutter-Dambuyant et al. 2006; Laubach et al. 2011; Ouwehand et al. 2011), dendritic cells (Bechetoille et al. 2007) and macrophages (Bechetoille et al. 2011) have been successfully incorporated into skin equivalent models. Nevertheless, the fact that skin equivalent is built using cells from multiple

| Table 1 | Advantages and limitations of the use of full-thickness skin models for anti-infective research (Grivel and Margolis 2009; Popov et al. 2014) |
|---------|-----------------------------------------------------------------------------------------------------------------------------------|
| **Full thickness skin model** | **Advantages** | **Limitations** |
| Human Skin Equivalent | Reproducibility | Lacks most dermal cellular components |
| | Control over the skin components | Genetic heterogeneity of the different skin components |
| | Possibility of genetic manipulation | | |
| Human Skin Explants | Preserved histology and architecture of the tissue | Interdonor variability |
| | Potential to recapitulate local immune responses | Limited time of tissue culture |
| | | Limited availability of the tissue |
donors makes the model genetically heterogeneous. On the other hand, the possibility of modulating the cellular components of the skin equivalents allows for studying the role of each cell type. Another advantage of skin equivalent models is the possibility of employing genetic engineering to unveil the function of a specific factor in the context of an infection and immune response, by inducing or depleting its expression in a particular cell type before its introduction into the skin matrix.

Skin explants preserve the histology and the complexity of the skin, being composed of several different resident cells (including immune cells) and appendages. This feature enables the study of the cutaneous local immune response raised against a pathogen or triggered by an anti-infective immune intervention at this site. However, skin explants have a short-term viability and limited availability. Additionally, the explant is not connected to circulation and consequently lacks the ability to recruit immune cells from the blood to the explant, such as neutrophils that are important for microbial clearance. Further, the inter-donor variability of the human skin explants makes interpretation of results more complex, thus requiring a greater number of samples from different donors to be analyzed in order to guarantee validity of the results. On the other hand, this feature is a key to better understand the host-inherent variability leading to differential individual responses, opening the door to personalized medicine.

3.2 Use of Human Skin Models in Anti-infective Research

Similar to the respiratory system, the skin has direct contact with the outside environment and therefore acts as a physical and chemical barrier for external threats. The skin is a common initial site of infection, where the first encounter of the immune system with a pathogen often occurs. Consequently, skin models are important for studying infections and anti-infective agents.

Although human skin models have been used to study microbial infection and pathogenesis by a number of authors, there are only a few studies focusing on anti-infective research. Since mouse skin does not resemble human skin in either histology or immunology (Pasparakis et al. 2014), efforts have been made to use human skin equivalent and human skin explant models to test safety and efficacy of antibacterial and antiviral compounds.

Poxviruses have a tropism for skin and mucosal tissues. Thereby, an organotypic culture system, built with human primary keratinocytes derived from neonatal foreskin, was used to prove the inhibitory effect of some acyclic nucleoside phosphonates on the viral cytopathic effect (Snoeck et al. 2002). The same model was also used to evaluate the antiviral activity of the compounds acyclovir, penciclovir, brivudin, foscarnet, and cidofovir against wild-type and mutant alpha-herpesviruses HSV type 1 and type 2, and VZV (Andrei et al. 2005). In both studies, the antivirals were added to the medium to mimic systemic delivery of the compounds.
Due to the fact that *Staphylococcus aureus* is the major cause of bacterial skin and soft tissue infections in humans (McCaig et al. 2006) and the widespread emergence of antibiotic-resistant strains, recent studies have tested strategies to block this pathogen at the initial site of the infection. A study performed in a human full-thickness skin equivalent, constructed by keratinocytes and fibroblasts seeded on top of a DED scaffold, showed that a tetraspanin-derived peptide can halve the number of *S. aureus* adherent to keratinocytes as compared to a scrambled control peptide, without impairing keratinocyte viability (Ventress et al. 2016). Although it is still not clear if *S. aureus* infection is the cause or the consequence of atopic dermatitis (AD), the low expression of cutaneous antimicrobial peptides (AMPs) in AD patients is thought to contribute to their increased susceptibility to infections (Ong et al. 2002). Therefore, different strategies have been employed to increase AMP levels to prevent *S. aureus* infection. A study published this year used heat-killed *Lactobacillus johnsonii* NCC 533, a non-replicating probiotic, as a topical treatment to increase the expression of AMP. This treatment reduced the adhesion of *S. aureus* to the RHE model by up to 74% (Rosignoli et al. 2018). Synthetic antimicrobial peptides derived from the natural AMP LL-37, namely P60.4, peptide 10, and SAAP-148, have been tested for their efficiency against multi-drug resistance strains on RHE models (Haisma et al. 2016), human skin equivalents (Haisma et al. 2014), and split-thickness human skin explants (de Breij et al. 2012), respectively. All peptides proved to have antimicrobial and antbiofilm properties against methicillin-resistant *S. aureus* (MRSA), and SAAP-148 was also effective against multi-drug resistant (MDR) *Acinetobacter baumannii* (de Breij et al. 2012). LL-37 formulated in a nano-sized liposome has also been proved to be effective against herpes simplex virus 1 (HSV-1) infection in RHE models (Ron-Doitch et al. 2016). Interestingly, the cytotoxicity induced by the formulation was greater in the monolayer of HaCaT cells than in the RHE model, which could be due to the presence of *stratum corneum*, acting as a protective layer.

Due to the increasing interest in this field of research, we focused on possible future directions with the skin model based on current trends in anti-infective research. The skin, in particular the dermis, can serve as an efficient delivery system of vaccines due to the easy access and presence of many immune cells which can elicit a strong response to vaccine antigens. Intradermal vaccination has already shown to alter Langerhans cell distribution within human skin explants (Pearton et al. 2010), as well as to induce DC maturation (de Grujl et al. 2006). Similarly, vaccination by microneedles can affect dendritic cell migration (Pearton et al. 2013). Since more evidence is emerging that the gut microbiome can influence immune responses, and hence vaccine efficacy (Littman 2017; Lynn and Pulendran 2017), one could argue that this applies to the skin microbiome as well. This hypothesis could be evaluated using the human skin model, whereby investigating the effect of known skin commensals on the cutaneous immune system. The application of topical compounds, e.g., in treating psoriasis (Kusuba et al. 2017) or other inflammatory diseases, and its effect on susceptibility to infections can be studied in depth with skin models, especially skin explants since the skin surface closely resembles the in vivo situation. In addition, novel anti-infective approaches,
such as nano-sponges that absorb secreted bacterial toxins (Hu et al. 2013; Escajadillo et al. 2017) or other innovative antimicrobial delivery systems (Hasan et al. 2017), can be tested for their efficacy with the skin model. Similarly, our group recently showed the protective effect of antibodies against *S. aureus* toxins within the context of the human skin explant model (Olaniyi et al. 2018).

Despite their many advantages, most of the currently used skin models have some limitations, as mentioned before. One of the most prominent limitations is the lack of a systemic flow, thereby leaving out essential immune processes which are key in anti-infective research, i.e., neutrophil recruitment, complement activation, and T cell migration (Pozzi et al. 2015). One way to overcome this deficit is the introduction of the organ on a chip technology, where microfluidics are incorporated in a culture model (Ahadian et al. 2017). Many skin-on-chip models have been established by using different cell types, as reviewed in (van den Broek et al. 2017), and may provide novel insights on therapeutic approaches in the future (Wufuer et al. 2016). Another technique to mimic skin perfusion is an isolated perfused human skin flap model, which has been developed to test drug delivery to the skin. In brief, a human skin explant was linked to an automated perfusion system via one vessel, thereby inducing a controlled, continuous flow. An infrared (IR) camera was used to constantly monitor the skin surface temperature, and thus the degree of skin perfusion (Ternullo et al. 2017). In conclusion, skin models provide a new platform to study the effectiveness of various novel anti-infective compounds, as summarized in Fig. 1.

**Fig. 1** Proposed future applications of human skin models in anti-infective research. a Human skin models can be used to study mechanism of action of intradermal vaccination and its efficacy against dermal pathogens and its interaction with commensal bacteria. b Novel topical agents may be tested for efficacy and potential side effects in a human skin model, either skin equivalent or skin explant. c Incorporation of microfluidics in the human skin model would mimic the systemic blood flow in the culture model enabling critical immune processes, such as neutrophil recruitment or tissue distribution of antibodies.
3.3 Conclusion

Taken together, over the last decade, human 3-D organotypic models have acquired increasing importance and great efforts have been made to improve the culture, handling, and reproducibility of these in vitro models. In contrast, a trend may be observed in which the use of animal models is becoming obsolete. One explanation could be the overall consensus that the use of animal models has to be reduced from an ethical point of view. Secondly, the occurrence of failed studies on novel anti-infectives in the clinical phase underlines the complexity of predicting human response using animal models. Despite the remaining limitations, the use of human organotypic models can be a valuable tool to facilitate the transition from pre-clinical to clinical studies.

3.4 Protocol: Culture of Human Skin Equivalents and Skin Explants for Use in Anti-infective Research

Figure 2 summarizes the procedures to culture full-thickness human skin models for use in anti-infective research.

Fig. 2 Schematic overview of the protocol for the culture of human skin equivalents and human skin explants. a Human skin equivalent can be cultured in two steps, starting with the dermal component consisting of collagen and fibroblasts. Other immune cells, such as macrophages, can be included at this step. To allow for the fibroblasts to contract, keratinocytes can be seeded after 7 days. Thereafter, differentiation of keratinocytes at air–liquid interface will require 7–14 days, depending on the origin of the keratinocytes. b When handling fresh human skin explants four different critical steps are involved. The first is the removal of the adipose tissue. To create a superficial wound for infection protocols, the stratum corneum can be removed by repeated tape-stripping followed by the preparation of punches with disposable surgical biopsy punches. The biopsy punches can be washed and cultured at air–liquid interface.
3.4.1 Procedure for the Preparation of Human Skin Equivalents

1. Materials

1.1 Cells
- Primary human dermal fibroblasts
- Human keratinocyte cell line/primary human keratinocytes

1.2 Reagents
- Collagen type I from bovine hides (3 mg/ml)
- Sterile phosphate buffered saline (PBS)
- Trypsin-EDTA (0.05%)
- 2N NaOH
- HBSS 10x with phenol-red
- Fibroblast culture medium:
  - DMEM High glucose
  - 100 U/mL of penicillin and 100 µg/mL of streptomycin
  - 10% fetal bovine serum (FBS) heat inactivated at 56 °C for 30 min
- Keratinocyte culture medium:
  - DMEM high glucose
  - 100 U/mL of penicillin and 100 µg/mL of streptomycin
- FAD medium:
  - DMEM/F12 (1:1)
  - 0.4 µg/mL hydrocortisone
  - 50 µg/mL L-ascorbic acid
  - 1 × 10^{-10} M Cholera toxin
  - 1% antibiotic-antimycotic
  - 10% FBS heat inactivated
- FAD airlift interface medium (ALI)
  - FAD medium
  - 0.5 mM Na-pyruvate
  - 1.6 mM CaCl2
  - 20 ng/mL TGF-alpha
  - 100 ng/mL GM-CSF

All media must be filtered and stored at 4 °C, no longer than 2 weeks.

1.3 Equipment
- Cell culture incubator (37 °C, 5% CO₂)
- Cell culture incubator (37 °C, 0% CO₂)
- Laminar flow class II cabinet
- Hemocytometer
• Pipet-aid
• Sterile disposable pipettes
• Single pipette with sterile tips
• Polypropylene tubes, 15 mL and 50 mL
• Glass Pasteur pipettes
• 12 transwell inserts preloaded in 12-well plates; pore size: 0.4 µm, sterile (Transwell®)
• Sterile filter bottles (0.2 µm)
• Ice buckets
• Water bath
• Centrifuge

2. Methods

2.1 Dermal component

Before starting the protocol:

• Place 10xHBSS, collagen and 2N NaOH on ice and disposable pipettes in the fridge;
• Set an incubator at 37 °C, 0% CO2;
• Calculate the amount of collagen mix needed to fill each insert with 500 µL. Considering that collagen mix is composed of collagen: 10xHBSS: fibroblast solution in the ratio 8:1:1, calculate the required amount of each of the components.

The following procedure must be performed under a laminar flow cabinet:

1. Harvest fibroblasts and count the number of cells using a hemocytometer
2. Suspend fibroblasts in FBS to a concentration of $1 \times 10^6$ cells/mL
3. Keep it at room temperature (RT)

From this step forward work always on ice:

4. Slowly add one part of chilled 10xHBSS to eight parts of chilled collagen in a 50 mL tube, with gentle swirling
5. Adjust the pH of the solution to pH 7.4 using 2N NaOH, dropwise until the color changes to orange/red

It usually requires 50 µL of 2N NaOH per 10 mL of collagen mix
6. Add one part of the fibroblasts solution to the collagen mix and homogenize the gel solution carefully, using a disposable pipette.
7. Add 500 µL of the complete collagen mix onto the filter inserts

Prevent bubbles by reverse pipetting
8. To allow the gel to solidify, place the 12-well plates for 1 h at 37 °C, 0% CO2
9. Add 2 mL FAD medium (1.5 mL outside, 0.5 mL inside the insert) and incubate for 7 days at 37 °C, 5% CO2. Replace the medium every 2-3 days.
2.2 Epidermal component

10. Harvest keratinocytes and count the number of cells using a hemocytometer
11. Prepare keratinocytes at $5 \times 10^5$ cell/ml in keratinocyte culture medium
12. Remove the medium inside the inserts
13. Add 20 μL of keratinocyte solution to the dermal equivalent
14. Incubate for 2 h at 37 °C, 5% CO₂
15. Add 500 μL of FAD medium inside the inserts
16. Incubate overnight
17. Remove all medium from the wells
18. Add 500 μL FAD ALI medium outside the insert, to allow culture at air–liquid interface
19. Incubate up to 7–14 days (dependent on differentiation status) and change FAD ALI medium every 2–3 days.

3.4.2 Procedure for the Preparation of Human Skin Explants (Olaniyi et al. 2018)

The outermost stratum of the epidermis, stratum corneum, is the major responsibility for maintaining the physical barrier function of the skin (Baroni et al. 2012). Therefore, this barrier has to be breached so that a pathogen can invade the tissue. Here, we described the use of the tape-stripping strategy as we consider that this method guarantees reproducibility between experiments.

1. Materials

1.1 Reagents

- Fresh human skin explants
- Sterile phosphate buffered saline (PBS)
- Culture medium:
  - Advanced DMEM supplemented with 4 mM L-glutamine

All media should be filtered and stored at 4 °C, no longer than 2 weeks.

1.2 Equipment

- Cell culture incubator (37 °C, 5% CO₂)
- Laminar flow class II cabinet
- Pipet-aid
- Sterile disposable pipettes
- Single pipette with sterile tips
- Glass Pasteur pipettes
- 12 transwell inserts preloaded in 12-well plates; pore size: 0.4 μm, sterile (Transwell®)
- Disposable surgical biopsy punches
Disposable scalpels
Sterile forceps
Sterile scissors
Sterile filter bottles (0.2 µm)
Water bath
Adhesive tape
Dissection board
Disinfected pins

2. Methods

2.1 Skin processing

Process the tissue immediately after its delivery.
The following procedure must be performed under a laminar flow cabinet:

1. Remove the excess of adipose tissue, using disposable scalpels or sterile scissors
2. Pin the skin in the dissection board
3. Strip the stratum corneum 30 times with an adhesive tape
   Make sure that the surface of the skin is dry before starting
4. Take 8 mm (in diameter) punch biopsies using disposable surgical biopsy punches
5. Place the punches inside a six-well plate, previously filled in with culture medium to prevent them to dry
6. Remove the remaining adipose tissue from the punches using a scalpel
7. Add 1 mL of culture medium in the 12-well plate
8. Place the skin punches in the inserts using sterile forceps
9. Culture the explants at air–liquid interface, at 37 °C, 5% CO₂.

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Authorship

Fabio Bagnoli had the initial idea of the topic, content, and structure of the review article. Astrid Hendriks and Ana Rita Cruz defined the content and wrote the manuscript. Fabio Bagnoli, Andrea Manetti, and Elisabetta Soldaini reviewed and wrote the manuscript. All authors approved the manuscript before it was submitted.

Declaration of interest

Astrid Hendriks and Ana Rita Cruz are Ph.D. fellows and are enrolled in the Infection and Immunity Ph.D. program, part of the Graduate school of Life Sciences at the University of Utrecht. Fabio Bagnoli, Andrea Manetti, and Elisabetta Soldaini are employees of GSK group of companies. Fabio Bagnoli owns patents on S. aureus vaccine candidates and GSK stocks. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
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