GAVIN PUBLISHERS

Journal of Vaccines, Immunology and Immunopathology

Case Report

Rijkers GT. J Vaccines Immunol: JVII-125.
DOI: 10.29011/2575-789X. 000025

The Crowning with Thorns: How an Intact Skin Forms the First Line of Defense Against Infections

Ger T. Rijkers[1,2*]

[1]Department of Sciences, University College Roosevelt, Middelburg, Netherlands

[2]Laboratory for Medical Microbiology and Immunology, St Antonius Hospital, Nieuwegein, Netherlands

[*]**Corresponding author:** Ger T. Rijkers, Department of Sciences, University College Roosevelt, P.O. Box 94 4330 AB Middelburg, Netherlands. Tel: +310118655500; Fax: +310118655508; Email: g.rijkers@ucr.nl

Citation: Rijkers GT (2018) The Crowning with Thorns: How an Intact Skin Forms the First Line of Defense Against Infections. J Vaccines Immunol: JVII-125. DOI: 10.29011/2575-789X. 000025

Received Date: 29 December, 2017; **Accepted Date:** 03 February, 2018; **Published Date:** 09 February, 2018

Introduction

The body's primary defenses against infection are mechanical barriers such as the skin and various mucous membranes. Like the skin, the mucous membranes of the airways, the digestive system and the urogenital system are in direct contact with the environment. Of these systems, the digestive tract has the largest area of contact with the microbial environment, approximately 200 square meters. The skin has a surface area of no more than 2 square meters. The mechanical barrier offered by the mucosal epithelial cells of the airways and the digestive tract is much less resistive than that of the skin, since the skin consists of several cell layers, with the topmost being an acellular horny layer. Therefore, there are only a few micro-organisms that are able to penetrate the intact skin, while it is relatively easy for them to pass through the more fragile mucous membranes. In addition to functioning as a physical barrier, the skin and mucous membranes have biochemical properties - e.g. low gastric pH, lysozyme in tears, fatty acids on the skin - that also make it difficult for microorganisms to survive at those places. The microbiota of the intestines and skin also constitute a biological barrier. This commensal microbiota use, a variety of mechanisms to make it harder for other - pathogenic or non-pathogenic - microorganisms to settle and then penetrate the body. In this review, the role of the various players (microbiota, host cellular and molecular components) and their mutual interactions in maintaining barrier integrity and homeostasis will be discussed.

A healthy skin is not sterile but forms the niche for a complex and dynamic ecosystem consisting of approximately 10^{12} micro-organisms, mainly bacteria, but also including fungi, and viruses [1] (Figure 1).

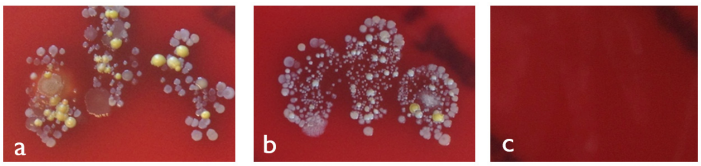

Figure 1: Skin microbiota. The provincial library of Zeeland in Middelburg, The Netherlands participates in the yearly national Science Weekend for primary school children. During a laboratory class on bacteria, children are asked to make a fingerprint on a petri dish with blood agar. Panel **a** show the culture of 3 fingers of a child who answered "yes" to the question: Who didn't wash their hands this morning? In panel **b**, another child was asked to first wash their hands with tap water and regular soap, air-dry and then make a print. In panel **c**, hands were cleaned with chlorhexidine (0.5%) in 70% ethanol first, then air dried before making a print. Agar plates were cultured overnight at 37°C.

The various skin sites can be classified into three microenvironments: sebaceous (glabella, alar crease, external auditory crease, retroauricular crease, occiput, manubrium, back), dry (volar forearm, hypothenar palm, buttock), and moist (nare, axillary vault, antecubital fossa, interdigital web space, inguinal crease, umbilicus, gluteal crease, popliteal fossa, toe web space, plantar heel) [2]. Overall, the skin microbiota is dominated by *Staphylococcus* spp (in particular *S. aureus* and *S. epidermidis*), *S. epidermidis* and *Corynebacteria*, which together form > 60% of the total population [2]. However, the differences in the microbiota occupying the various skin sites is enormous and have been described as *"*ecologically dissimilar as rain forests are to deserts*"* [3]. Skin microbiota composition thus is influenced by body region, by biological sex, age, health status, geographical location, ethnic background, depth of the skin, use of cosmetics

and antibiotics, as well as life-style factors such as pet ownership and alcohol consumption [4,5].

The commensal skin microbiota contributes to the protection of the human host against infections in many different ways (see reference [1] and (Table 1) for an overview). Commensal skin microbiota competes with (potential) pathogens for an ecological niche. Commensal bacteria can also directly inhibit the growth of pathogens: lipoteichoic acid in the cell wall *S. epidermidis* inhibits the growth of *Propionibacterium acnes* [6]. The cutaneous fungus *Malassezia* produces azelaic acid by enzymatic degradation of external lipids and thus contributes to the low pH of the skin [7]. Via several mechanisms, skin microbiota stimulates the innate and acquired immune system of the skin (Table 1). In experimental animals it has been demonstrated that colonization with *S. epidermidis* is sufficient to confer effective T cell immunity against *Leishmania major*, via induction of IL-1 and IL-17 [8] Based on [9].

Inhibition of growth of pathogens	Competition for ecological niches, Inhibition of growth
Interaction with skin tissue and cells	Maintenance of epidermal integrity, Keratinocyte homeostasis, Keratinocyte cortisol production
Interaction with the skin immune system	Stimulation of innate immunity (including antimicrobial peptides and cytokines), Induction of regulatory T cells, Production of regulatory cytokines (IL-10, IL-17)

Table 1: Interaction between commensal skin microbiota and the human host.

The Skin Forms an Effective Barrier Against Infection

An intact skin is an effective anatomical barrier against the ingress of microorganisms. The skin consists of a number of cell layers of epithelial cells with the stratum corneum being the outermost layer (Figure 2).

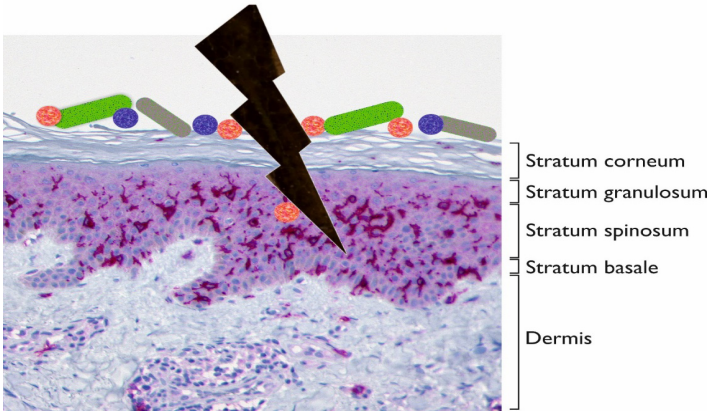

Figure 2: Breaching the skin barrier. Microbiota on the skin are schematically indicated. Langerhans cells in the stratum spinosum are stained with CD1d antibodies and visualized by immunohistochemistry. Photograph courtesy Dr. H. Kutzner, Friedrichshafen, Germany. Thorn taken from Jheroniumus Bosh Crowing with Thorns (see also Figure 3).

Figure 3: Fragment of (a follower of?) Jheronimus Bosch The Crowning with Thorns (Museo Provincial de Bellas Artes, Valencia) The infrared reflectrogram of the painting shows that the in the underlying prestudy the crown of thorns has caused substantially less tissue damage than in the ultimate painting itself.

The stratum corneum is the final stage of a complex and tightly regulated cellular differentiation process called keratinization. The cells in the stratum basale serve as the stem cells and as the keratinocytes divide they move up into the stratum spinosum. The cohesion among the keratinocytes in the stratum spinosum is provided by intermediate keratin filaments. When keratinocytes

mature they accumulate keratohyalin granules and lamellar bodies, and form the third layer: the stratum granulosum. The keratohyalin granules contain filaggrin, which binds to keratin tonofilaments. This is the soft keratin of the skin. In the stratum corneum, the keratinocytes have lost all organelles, including the nucleus, are flattened, and are now termed corneocytes. The cell membrane has been replaced by a cornified envelope. The lamellar bodies have secreted lipids, which fills the spaces between corneocytes. The extracellular lipids anchor on the cornified envelope and thus form an important physical barrier in the stratum corneum. In the upper stratum corneum, filaggrin is enzymatically dissociated from keratin and further metabolized. Two filaggrin metabolites, urocanic acid and pyrrolidine carboxylic acid, contribute to skin integrity. Urocanic acid is an UV absorbing molecule and also contributes to maintenance of the low pH of the skin. Pyrrolidine carboxylic acid contributes to moisturizing of the stratum corneum [10]. The importance of filaggrin in skin barrier integrity is demonstrated by the finding that loss of function mutations in the filaggrin gene are strongly associated with atopic dermatitis [11] and ichthyosis vulgaris [12].

A Whole Range of Anti-Microbial Proteins Prevent Skin Infections

In addition to the physical and cellular barriers discussed above, there are > 1000 proteins with an anti-microbial action that are produced in the skin and/or are present in secretions (saliva, tears, mucus, sweat, breast milk). These components can either kill micro-organisms directly or cause their growth to be greatly delayed or have other effects that support the immune response (see the Antimicrobial Peptide Database. Anti-microbial proteins not only play a role in the prevention of infections, but also when micro-organisms, despite all obstacles, have penetrated through the epithelium. Examples of such proteins are lysozyme, lactoferrin, cationic peptides (defensins and cathelicidins). Interferons and factors of the complement system also have anti-microbial effects, but they are, due to space restrictions, not discussed in this paper. This whole arsenal of anti-microbial proteins is an important component of the innate immune system. The most important of these proteins will now be discussed successively.

Lysozyme and Lactoferrin

Lysozyme, first described by Alexander Fleming in 1922 [13], is an enzyme that is able to break down carbohydrate chains in the cell wall of bacteria so that the cell wall loses its structural integrity. This enzyme is present in almost all secretions (mucus, saliva, tears, mucus, sweat, breast milk) where it is secreted by epithelial cells [14]. Lysozyme is also found in granules of neutrophilic granulocytes where it contributes to the degradation of phagocytic micro-organisms. The protein lactoferrin is an iron-binding protein which, like lysozyme, is produced by epithelial cells and released to secretions. The highest concentration of lactoferrin is found in breast milk. Lactoferrin is also an important component of the granules of the neutrophil granulocytes and can be secreted locally by these cells. Because of the iron-binding effect lactoferrin can inhibit the proliferation of bacteria that need iron for their growth. In addition, this protein also has a direct bactericidal effect because it destabilizes the bacterial cell membrane. It can also bind to lipopolysaccharide (LPS, endotoxin), a component of the cell wall of Gram-negative bacteria [15]. Lactoferrin is mainly produced by mucosal epithelial cells and is present in most body fluids, in particular breast milk [16]. Lactoferrin has an indirect effect on skin immunity as evidenced by the beneficial effects of oral administration of lactoferrin in psoriasis and other skin diseases [17].

Cationic Anti-Microbial Peptides: Defensins and Cathelidicins

Cationic anti-microbial peptides are small positively charged proteins, about 30-50 amino acids in size. Many of these amino acids in such anti-microbial peptides are the positively charged arginines and lysines. The defensins form the largest group of these peptides. On the basis of structural differences, two classes of defensins can be distinguished, α-defensins and β-defensins. The β-defensins (HBD) are subdivided into HBD-1, -2, -3, and -4. The different types of human β-defensins are mainly produced and secreted by different types of epithelial cells (skin, lungs, intestine, kidney, prostate, epididymis, etc.) [18]. Depending on the type of β-defensin, its production may be constitutive and/or induced by activation with microorganisms (e.g. rhinovirus, HIV or lipopolysaccharides from bacteria) or certain cytokines [19,20]. Both classes of defensins have anti-microbial activity against a wide range of bacteria, fungi, chlamydiae and envelope viruses. The action of these cationic (positively charged) anti-microbial peptides relies mainly on their ability to interact with the surface of the micro-organism. This interaction is followed by insertion of the peptide into the cytoplasmic membrane that usually contains negatively charged phospholipids. This makes the membrane of the micro-organism porous and loses its vitality. Other anti-microbial mechanisms of action of defensins are also known. Thus, by their interaction with the viral membrane, defensins can prevent the virus from fusing with the endosome of the host cell, thereby preventing virus replication [21]. Some micro-organisms have built up a defense against these anti-microbial peptides. Examples of these are modifications of lipopolysaccharides occurring in the cell wall, as a result of which they receive a less negative charge or the presence of membrane-bound proteases which can degrade the cationic peptides [22,23].

In addition to a direct anti-microbial activity, α and β defensins also possess important immunoregulatory properties [18]. Thus, α and β defensins have a chemotactic effect on monocytes, (immature)

dendritic cells and T lymphocytes (ie they pull cells to the site of the infection) and they can promote the production of cytokines by monocytes and epithelial cells. Cathelicidins form another group of cationic peptides. These differences in molecular structure and in biological properties of the defensins. The only known human cathelicidin, LL-37, is constitutively produced by a large number of different types of epithelial cells (including skin, gastrointestinal tract and airways, testis, sweat glands) and by virtually all types of leukocytes, but mainly by neutrophilic granulocytes. Like defensins, LL-37 has, besides its direct anti-microbial action, several other functions, such as chemotaxis, wound healing and blood vessel formation (angiogenesis), degranulation of mast cells and activation of chemokine secretion. LL-37 is particularly present in high concentrations in seminal fluid and in breast milk. In the human skin, keratinocytes are the main cellular source of AMPs (Table 2), but also mast cells, neutrophils, sebocytes and eccrine epithelial cells are able to synthesize antimicrobial peptides [18].

AMP	Primary expression	Reference
Human β defensin-1	Breast, abdomen	[24]
Human β defensin-2	Genitals, cheeks	[25]
Human β defensin-3	Forehead	[26]
S100A7 (psoriasin)	Head, elbows, hand palms	[27]
RNAse 7	Arms, chest, abdomen	[28]
LL37	No preference location	[29,30]

Table 2: Antimicrobial peptides expressed by skin keratinocytes.

Data on expression at various skin sites taken from reference [31].

The Innate and Acquired Immune System of The Skin

The immune system of the skin consists of both an innate and an acquired part. The major cellular players are indicated in (Table 3).

	Innate immune system	Acquired immune system
Epidermis	Keratinocytes, Langerhans cells	
Dermis	Fibroblasts, Macrophages, NK cells,Innate lymphoid cells (ILC), Mast cells, (Neutrophils)	(plamacytoid Dendritic cells and dermal Dendritic cells), Cytotoxic T cells, Thelper (Th) 1 cells, Th2 cells, Th17 cells, resident memory T cells, B cells

Table 3: Cells of the innate and acquired immune system in the skin.

The innate immune system is constitutively present and can act immediately upon contact with a pathogen. The acquired immune response will take time to develop and become operative. During an acquired immune response, next to effector cells and molecules, also specific memory cells will be generated as will be detailed below.

In the epidermis, the keratinocytes and Langerhans cells are cellular components of the innate immune system. Keratinocytes serve an important role in the initiation of an innate immune response because they have the ability to recognize invading pathogens via Toll like receptors [32,33]. Upon activation, keratinocytes produce a wide range of cytokines that either directly inhibit the pathogen or indirectly activate other components of the skin immune system [34]. The Langerhans cell in the stratum spinosum of the epidermis is the first professional antigen recognizing and -processing cells of the skin immune system (Figure 2). Langerhans cells recognize invading pathogens via Toll-like receptors, capture the pathogen via endocytic pathways [35]. The cells of the acquired immune system are mainly located in the dermis of the skin (Table 3). Based on reference [1].

The skin, also a healthy, non-inflamed skin, contains a (surprising) large pool of T lymphocytes. This are mostly memory T cells expressing the skin-homing marker CLA (cutaneous lymphocyte antigen) [36,37]. Naïve T lymphocytes, upon successful activation by antigen differentiate into effector and memory cells. During this process they start expressing CTLA. CTLA is a trafficking receptor which is expressed on up to 15% of T lymphocytes in peripheral circulation, but on virtually all skin infiltrating memory T lymphocytes. The memory T lymphocytes in the skin belong to the category of so-called resident memory T cells (T_{RM}). T_{RM} remain present in the skin after the infection has cleared and are ready to act immediately upon antigen re-encounter. T_{RM} in the epidermis are $CD103^+$ (a receptor for E-cadherin on epithelial cells) and are high producers of IFN-γ and TNF-α. The T_{RM} which reside in the dermis are $CD103^-$ and have a lower effector function [1]. Next to the T_{RM} cells, up to 5-10% of the T cells in the skin are $FOXP3^+$ regulatory T cells (T_{reg}). T_{reg} cells are of vital importance for regulation of the immune response, in particular in maintaining the balance between Th1 and Th2 cells. Th17 cells (which secrete the IL-17 cytokine) are also found in the skin. Th17 cells provide immunity against a variety of pathogens, including *Candida albicans*. Overactivity of Th17 cells leads to inflammation, such as is the case in psoriasis [38].

The classical distinction between innate and acquired immunity as indicated in Table 3 becomes blurred by the recent discovery of a novel type of cells, the so-called innate lymphoid cells (ILCs). ILCs are innate immune cells belonging to the lymphoid lineage. They do not express antigen-specific receptors [39]. ILCs were first discovered in the intestine, where they contribute to epithelial barrier integrity [39]. ILCs now have been found also to be a component of the skin immune system [40],

in particular type 2 ILCs [41,42] and type 3 ILCs [43]. Type 3 ILCs are producers of IL-17A cytokines, and thus can contribute to inflammatory responses in the skin, such as found in psoriasis [43].

Disruption of The Skin Barrier

The skin barrier can be disrupted by a knife such as in the patient described in the clinical case or by a thorn as illustrated in (Figure 3). As a result, bacteria, present on the knife, on the thorn, as well as bacteria present on the skin, penetrate the body and will be subsequently phagocytized by resident macrophages [44]. Among other things, the pro-inflammatory cytokines cause the keratinocytes at the locus of infection to express more adhesion molecules. Neutrophilic granulocytes gather, exit the bloodstream, reach the bacteria at the site of infection via a gradient of chemokines. This causes the acute local inflammation with its clinical signs of pain (dolor), swelling (tumor), heat (calor), redness (rubor) and loss of function (functio laesa). Under the influence of these same cytokines, specialized antigen-presenting skin cells in the skin, the so-called Langerhans cells (Figure 2) take up the bacteria and transport bacterial antigens to the regional lymph node - in the present clinical case, the axillary lymph node. Once there, they induce an adaptive immune response, which explains the swollen lymph node in the armpit. As part of the local inflammatory response, large numbers of neutrophils are activated. These granulocytes not only absorb bacteria through phagocytosis but also secrete lytic enzymes and reactive oxygen metabolites, leading to local tissue degradation. Pus is the result of tissue and granulocyte necrosis. An incision allows this necrotic material to be discharged. Most of the live bacteria will have been eliminated by then and the wound can heal. A small scar will remain, however. The adaptive immune response (axillary lymph node) will abate, primarily because there are no more antigens left to support it.

Interplay Between Skin Microbiota, Skin Tissues and Skin Immune System in Ensuring Optimal Barrier Integrity

The skin is the primary interface with the external environment, full of pathogens, UV radiation, allergens and chemical irritants. The skin therefore serves as a mechanical, biological and immunological barrier in order to protect the host from an undesirable insult. For the optimal functioning of this barrier, the mechanical, biological, and immunological components are interconnected and mutually regulated. In the preceding sections already examples have been given of how commensal micro-organisms on the skin regulate barrier functions [45]. Important to realize is that an overactive skin immune system, especially Th2 immune reactions, can have a negative effect on the skin barrier [46]. The Th2 cytokines IL-4 and IL-13 have a negative effect on the skin barrier function because they downregulate the production of filaggrin and keratins and of the cornified envelope components. IL-31, which is another Th2 cytokine and IL-33 also downregulate filaggrin expression [47,48]. These findings underscore the importance of a closely regulated interplay between the immune system of the skin and its external stimuli in order to protect against infections while maintaining internal homeostasis.

Clinical Case: Penetration of The Skin Barrier

A 45-year-old gardener was pruning bushes in the garden of one of his clients. He accidentally cut him-self with his trimming knife, which had soil and bark on the blade. It was a substantial cut in the left index finger. He used his handkerchief to stop the bleeding and continued working. However, in the evening he noticed that his left index finger was throbbing, felt warm to the touch, had a reddish appearance and was painful. It was also slightly swollen. He did not sleep well that night due to this discomfort. Next day the swelling increased. In the evening, he also felt a small, tender swelling in his left arm pit. The following day, the swelling was even more pronounced and felt somewhat flaccid. The man went to see his GP, who observed that the subcutaneous swelling had a yellow shine and was fluctuating. He subsequently used a lancet to make a small incision from which creamy yellow pus flowed out. This immediately alleviated the pain. Within days the wound had healed completely. The swelling in the armpit also gradually disappeared.

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