Old Herborn University Seminar Monograph 28:
The epidermis of man: co-existing with commensals.
Editors: Peter J. Heidt, Pearay L. Ogra, Michael Zasloff, and Volker Rusch.
Old Herborn University Foundation, Herborn, Germany: 33-42 (2015).

OUR MICROBIAL SELF: ESSENTIAL FUNCTIONS FOR COMMENSAL BACTERIA ON THE SKIN

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INTRODUCTION

It is increasingly well accepted that the community of microbes that normally inhabit our body influences human immune behaviour, but it remains incompletely understood how this occurs or how this information can be used therapeutically. We have been particularly interested in the analysis of potentially mutually beneficial relationships between the most commonly isolated skin bacterium, *Staphylococcus epidermidis*, and the skin. Interactions between *S. epidermidis* and our immune system enhances skin protection by suppressing uncontrolled inflammatory reactions and stimulates host antimicrobial peptide (AMP) production (*Lai* et al., 2009, 2010). These specific beneficial effects on immunity are now just two of several actions that have been associated with the presence of a normal microbiome (*Wanke*, 2011; *Naik* et al., 2012). However, it was unclear how cutaneous microbes can exert such effects while residing on top of the epidermis, not in contact with living cells. To address this important question, we have recently shown that low numbers of bacteria penetrate the intact epidermal barrier in mice and in humans (*Nakatsuji* et al., 2013). This means that the skin acts as a filter (rather than a barrier), and controls the balance of the dermal microbial communities. In this paper we suggest that the presence of microbes below the surface of the skin can provide a rational explanation for how microbes influence normal skin homeostatic functions. In particular, we propose that the pathophysiology of several skin diseases can be explained by disruption of balance between the barrier and the microbiome.

COMMENSAL MICROBES MAINTAIN SKIN HOMEOSTASIS

Several recent studies have shown that the skin commensal bacterial community modulates the host immune system. In a 2009 publication in *Nature Medicine*, our group first demonstrated a beneficial interaction between *S. epidermidis*, a predominant commensal species on normal human skin, and skin inflammatory responses (*Lai* et al., 2009). We showed that a unique lipoteichoic acid (LTA) produced by *S. epidermidis* was recognized through a MyD88-dependent process to activate TNF-a receptor–associated factor-1 (TRAF1). This event inhibited excess skin inflammation during skin injury. In separate but related work, we also demonstrated that a small molecule of <10 kDa secreted from *S. epidermidis* increased expression of human β-defensins (hBDs) in murine skin or human keratinocytes (*Lai* et al., 2010).

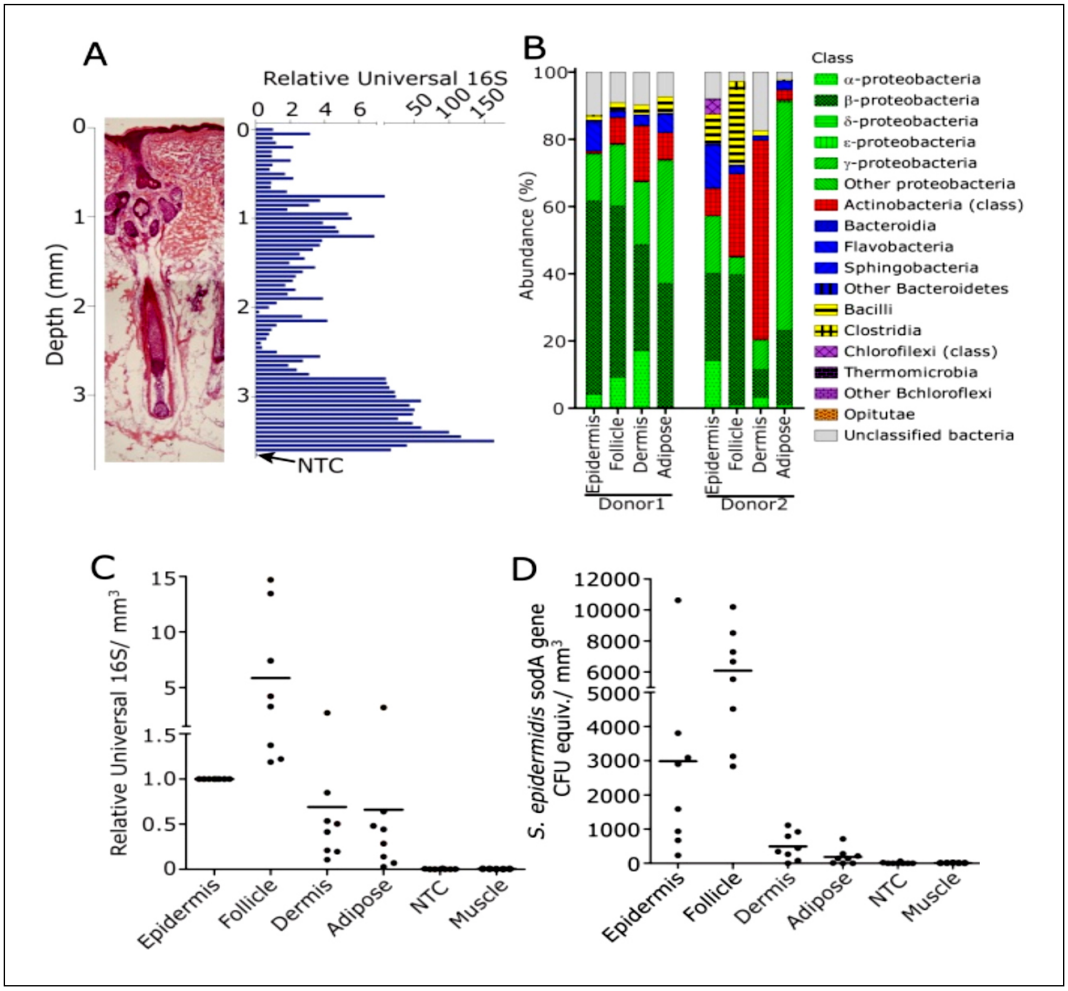

Figure 1: Bacterial DNA is present in normal human dermis. (Taken from: Nakatsuji et al., 2014). **(A)** 16S rRNA quantified with real-time qPCR from total DNA extracted from sequential 50 µm horizontal sections of human facial skin, H&E staining of the sectioned biopsy is shown in the same scale as the Y-axis of the graph. **(B)** Pyrosequencing for diversity analysis in Laser Capture Microdissection (LCM) sections of isolated epidermis, follicle, dermis and adipose tissue. Bacterial class is identified by colour. 2 representative patients are shown. **(C)** qPCR for total bacteria by universal 16S rRNA gene from LCM samples as prepared in (B) (n=8). **(D)** qPCR for *S. epidermidis* from LCM of same as (C). NTC=non-tissue control from same slide of LCM. Muscle is additional normal human tissue control that shows no bacterial 16S signal is detected in this tissue processed along slide of skin biopsy.

Following our discoveries, other groups also have shown that a product secreted from *S. epidermidis* strongly sensitizes the host innate immune response to pathogens (*Wanke* et al., 2011). In cultured human keratinocytes, activation of TLR2 by peptidoglycan, the major constituent of Gram-positive cell wall, strengthens the cellular tight junctions (*Yuki* et al., 2011). More recently, it was demonstrated that skin commensal bacteria enhanced cutaneous T-cell functions via activation of IL-1 signalling (*Naik* et al., 2012). Indeed, germ-free mice are more susceptible to infections in the

skin than conventionally housed mice (*Naik* et al., 2012). Furthermore, additional work has suggested that a shift in the microbiome will be detrimental not only because of the loss of beneficial species, but also because of the addition of pathogenic species. Colonization of mice with *S. aureus* producing delta-toxin led to exacerbation of Th2 response and elevated IgE resembling AD (*Nakamura* et al., 2013). This response was dependent on mast cells residing in the dermis. A more detailed review of this topic can be found in a recent review (*Sanford* and *Gallo*, 2013).

THE EPIDERMIS IS A MICROBIAL FILTER, NOT A BARRIER

Inherent to the observations described above is the conclusion that bacteria must somehow interact with the cells of the immune system to subsequently influence their action. However, this rational conclusion contradicted the prevailing belief that the structure of the skin's stratum corneum, and tight junctions between epithelial cells, prevent entry of bacteria under normal conditions. In fact, topical drug delivery is severely limited by the size of molecules, with a typical upper limit of entry to be only about 500 Daltons. The question remained then: how does the microbiome exert control on cells located below the epidermis?

The obvious answer to this question was that the epidermis was not absolute barrier to bacteria or products of the microbiome that can diffuse to deeper levels. To determine if bacteria enter the dermis we first made sequential horizontal sections of normal human facial and palm skin (bearing no hair follicles) and determined abundance of microbial DNA at different depths with qPCR using universal 16S rRNA primers (*Horz* et al., 2005) (Figure 1A). The existence of bacterial 16S rRNA genes was seen deep in subcutaneous tissues. In contrast, the qPCR signal was negative in simultaneously-prepared control samples from OCT tissue embedding compound surrounding the tissue, and downstream of cutting by the cryostat blade, [non-tissue control (NTC)]. These indicated that the detection of the bacterial 16S rRNA gene was not due to contamination from reagents, sample processing and sample crosstalk. Notably, however, 16S rRNA genes were detected in skin samples beneath the maximal depth of follicles in facial skin and below the eccrine glands in palm skin. Appendageal structures were expected to contain a microbial community, but our first experiments suggested that bacterial DNA may exist outside of the follicle or sweat glands. Subsequent studies using a laser capture microdissection (LCM) technique to isolate dermis away from appendages confirmed the existence of microbes in dermis and adipose. We also used the combination of LCM and pyrosequencing and real-time qPCR with 16S rRNA primers or genus- and species- specific primer/probes to quantify and identify the bacterial DNAs existing in each skin compartment. Universal 16S rRNA and *S. epidermidis*-specific genes were consistently detected by real-time qPCR in LCM-isolated sections from dermal and adipose tissue, (Figure. 1B,C, D). To rule out that the qPCR signals from these regions were due to contamination of these samples, NTC samples were prepared by LCM in the same manner as the pyrosequencing analysis. In addition, LCM section of human skeletal muscle biopsies served as another control. We also used multiple

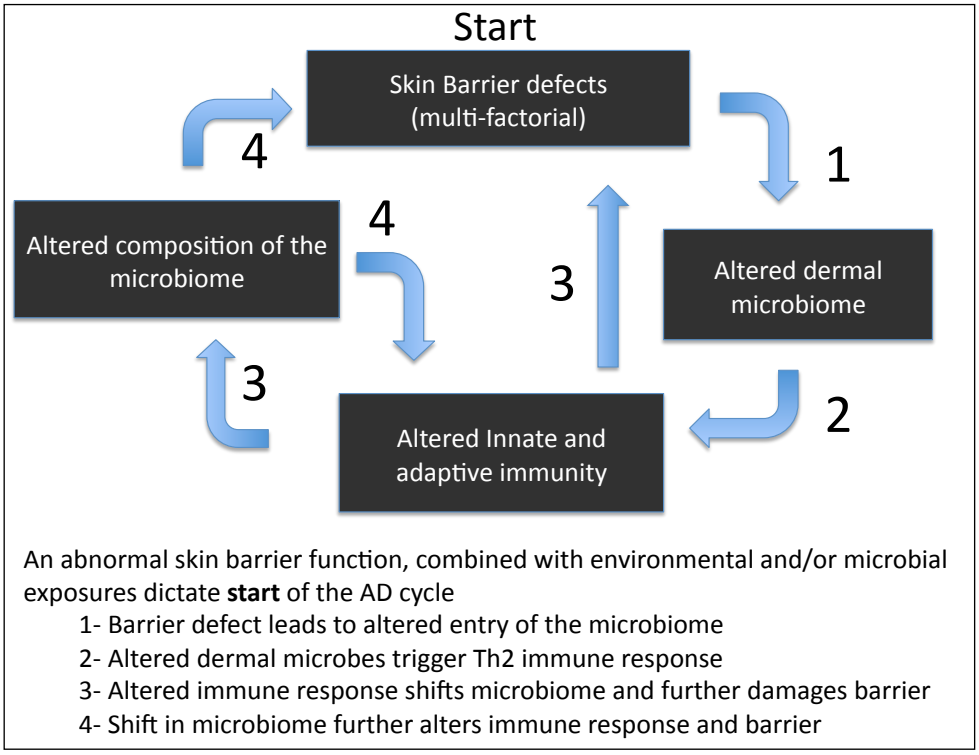

Figure 2: A model to connect the function of the skin barrier in Atopic Dermatitis (AD) with the function of the microbiome

other techniques to further confirm the results. Due to space limitations we will not show the data here. To summarize: bacteria were also detectable by Gram-staining inboth reticular dermis and dermal adipose, by immunostaining for *S. epidermidis*, *Pseudomonas* spp. and anti-lipopolysaccharide (LPS). Also, co-immunostaining with anti-CD11c failed to detect these bacterial products within these cells by confocal imaging, indicating that the presence of bacteria in the dermis was not associated with classical phagocytic immune cells that could have engulfed bacteria in transit from the surface into dermis. Bacterial 16S rRNA was also detectable by *in situ* hybridization with an oligonucleotide probe EUB338 (*Amann* et al., 1990) in dermal adipose tissue. In summary, we have optimized several independent techniques (qPCR for horizontal sections, Gram stain, several immunostains, *in situ* hybridization, LCM and pyrosequencing, and LCM and qPCR) to detect bacterial RNA, DNA and antigens in the dermis.

A MODEL FOR THE LINK BETWEEN HOST BARRIER DEFECTS AND THE MICROBIOME

Patients with atopic dermatitis (AD) have known defects in both physical and antimicrobial barriers. Therefore, if these barrier defects influence microbe entry into the skin, and the presence of microbes in the dermis alters skin im-

mune responses (as our data suggest), our observations provide an explanation for how barrier defects may result in abnormal inflammation. This is not infection, but rather a change in an important environmental signal. Such an altered signal could perpetuate a chronic cycle: an inherent barrier defect can lead to an altered microbial community causing altered immunity, altered immunity may then alter the surface microbiome and barrier, and the dysbiosis of the dermal microbial community then may perpetuate the disease by further harming the barrier and triggering inflammation. An illustration of our hypothesis for the interaction between these variables is seen in Figure 2. The significance of this discovery for human disease is that our model explains how several different genetic or environmental differences in barrier function may result in dysbiosis and skin inflammation.

A fundamental assumption of this model is that the presence of bacteria, or bacterial products, in the dermis is a critical factor in both the acute response (steps 1-3) and the chronic responses (steps 3-4). Although there is good evidence now that the microbiome influences immunity there has not been adequate work to show how this links to the known physical barrier defects in AD. Furthermore, there is increasing evidence that specific members of the microbiome (in particular *S. aureus*) may exacerbate the allergic response (*Nakamura* et al., 2013). Therefore, it is important to understand mechanisms that regulate the entry of microbes into the skin and explore how this influences the immune response. Furthermore, showing which bacteria can best minimize *S. aureus* colonization and suppress allergic disease may be of great clinical importance.

The concept of a dermal microbiome, and the proposed link between barrier and the immune response characteristic of AD, suggests that interactions take place between microbes and live cells below the epidermal barrier (Figure 2). Our current hypothesis extends this and suggests that the dysbiosis of the skin surface microbiome alters the immunological phenotype by penetrating into the dermis. This hypothesis does not necessarily disprove the notion that the dermis is sterile. The dermal microbiome may or may not be alive. The critical prior finding is that the products of microbes (bacterial 16S rRNA, multiple species-specific genomic DNA and antigens) are located in contact with live cells below the stratum corneum. Notably, the microbial products observed deep in the skin are generally not present within classical $CD11c^{+}$ phagocytic immune cells, suggesting the microbes enter across the epidermal barrier rather than carried in by phagocytosis. The capacity of animal models to experimentally manipulate dermal microbe entry permits us to test how this affects skin immunity. Such observations can provide an important step towards explaining the association between skin barrier defects and inflammation in AD.

A CHANGE IN THE BARRIER RESULTS IN A CHANGE IN DERMAL MICROBES

Loss-of-function mutations found within the filaggrin gene (*FLG*) represent a significant genetic factor predisposing to AD (*Palmer* et al., 2006; *Smith* et al., 2006; *Sandilands* et al., 2007; *Bisgaard* et al., 2008). It was

thought that *FLG* mutations facilitate penetration of environmental pollutants, irritants, and allergens passing the epidermal barrier, resulting in development of uncontrolled allergic reactions (*Bisgaard* et al., 2008; *Fallon* et al., 2009).

In preliminary studies we used *FLG* null mutants in a Balb/c background ($FLG^{ft/ft}$ Balb/c), which have been bred to remove the matted mutation (*ma*) that also was present in the originally described flaky-tail mice. This mouse is a specific *FLG* mutant, and has no spontaneous phenotype. Prior work has shown that the $FLG^{ft/ft}$ mouse will develop an AD-like phenotype if manipulated by external injury from tape-stripping, and an immunological stimulus from repeated cutaneous ovalbumin (OVA) stimulation (*Fallon* et al., 2009). Therefore, we performed a preliminary experiment to study microbe entry under each condition. We found that the $FLG^{ft/ft}$ Balb/c mouse has enhanced barrier defect as classically measured by transepidermal water loss (TEWL). This correlated with increased penetration of *Staphylococcus aureus* into the dermis measured by a newly developed technique of tracking entry of a candidate microbe applied to the surface. *S. aureus* was only slightly increased on the surface in $FLG^{ft/ft}$ Balb/c or in comparison to PBS-sensitized group, (all groups were tape-stripped). Notably, *S. aureus* was greatly increased in sub-epidermal dermal and adipose tissue of OVA-sensitized $FLG^{ft/ft}$ skin compared to identically treated WT. These data support our working hypothesis.

ANTIMICROBIAL PEPTIDES ARE AN IMPORTANT COMPONENT OF THE EPIDERMAL BARRIER

Our labs work over the past 20 years has shown how AMPs are an integral part of the host innate immune system. The secretion or release of these peptides provides direct disinfecting action against various infectious pathogens. Critical roles of AMPs on the skin innate immune defence have been shown by our group using cathelicidin knockout mice ($Camp^{-/-}$). We discovered that these mice were susceptible to pathogen infections, thus showing how important AMPs are in mammalian immunity (*Nizet* et al., 2001). Subsequently, we and others found that the induction of some AMPs such as cathelicidin and hBD-2 and hBD-3 is low in AD (*Ong* et al., 2002; *Nomura* et al., 2003; *Lande* et al., 2007). This diminished induction of AMPs linked to a higher propensity to *S. aureus* colonization and may explain why infections occur more frequently in AD. Similar to findings in the *Flg-/-* mouse we saw a significant difference in the *S aureus* in dermal and adipose compartments between $Camp^{-/-}$ and WT. This suggests *Camp* participates as part of the filter regulating dermal microbial abundance.

CLINICAL EVIDENCE FOR AN ABNORMAL MICROBIAL BARRIER IN ATOPIC DERMATITIS

In addition to our new data in mouse models, we have also obtained preliminary clinical data from patients that support our hypothesis. A pilot study using lesional and non-lesional forearm skin from patients with AD demonstrated important differences in the dermal microbial community of AD. In

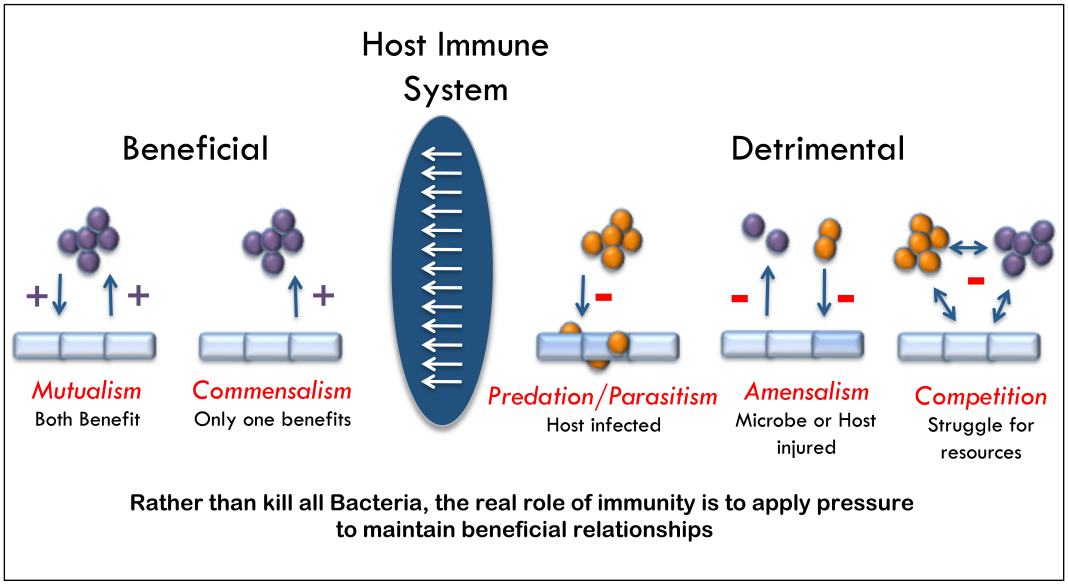

Figure 3: Potential relationships between microbes and us: A new way to think about the immune system. (Adapted from: Schommer and Gallo, 2013).

non-lesional skin of AD, the microflora in the epidermis and dermis primarily consisted of the phyla of *Proteobacteria* and/or *Actinobacteria*. Importantly, the diversity in the dermis of non-lesional AD skin was different that seen in any prior analysis from normal patient skin. Also, we measured a higher relative abundance of bacteria in AD lesional compared to normal skin or non-lesional skin. In lesional skin, proportions of *Firmicutes* were higher in the epidermis of lesional sites than non-lesional sites, a finding consistent with previous observations of the superficial community of AD skin and the increased abundance of *S. aureus* (*Kong* et al., 2012). Furthermore, we found a similar trend in the bacterial communities in the dermis in AD as in the epidermis. Real-time qPCR analysis indicated that *S. aureus* (a prominent member of the *Bacilli* class) was detected both in epidermis and dermis of lesional skin, but not in non-lesional skin**.** In addition, total bacterial abundance in the dermis of lesional skin was much higher than that of non-lesional skin. We also detected increased level of *Propionibacterium acnes* (an *Actinobacteria*) in lesional sites, and the level was higher in dermis than epidermis. (Of note: the skin surface is cleaned for standard surgical prep prior to biopsy and was not clinically infected).

CONCLUSIONS

In summary, rapidly advancing knowledge about the composition and function of the microbiome is redefining conceptions about our immune system. Rather than a permanent hostile relationship, we probably co-exist in a mutualistic relationship. Under such a relationship, both parties benefit. Of course, this is not the only possible interaction between microbes and the

host, and all of the commensal bacteria that have been described to benefit us can also harm us. Figure 3 presents a summary of the types of relationships that can exist between host and microbe. This figure also illustrates our new concept for the function of the immune system. Given our current knowledge regarding the functions of the microbiome, it is more reasonable to consider the host immune system as a mechanism to apply pressure to maintain beneficial relationships between bacteria and the host. In fact, given the abundance and distribution of bacteria both at the surface and within us, it is most logical now to consider the human body as a heterogeneous collection of organisms hoping to work towards a common good. Thus, the concept of **the microbial self** emerges from the paradigm-shifting observations that bacteria enter deep into the dermis. We hypothesize that this event is central to how the microbiome exerts influence on the immune system, and that the entry of some bacteria will exacerbate disease while others may rescue it. The near future will bring new insight into how the combination of epithelial function and the skin microbiome influences skin immunity. The implications of this are great, and hopefully will provide new therapeutic approaches to human disease.

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